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Development of a system for genetic manipulation of Bartonella bacilliformis

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Development of a system for genetic manipulation of *Bartonella bacilliformis*.

By

James M. Battisti

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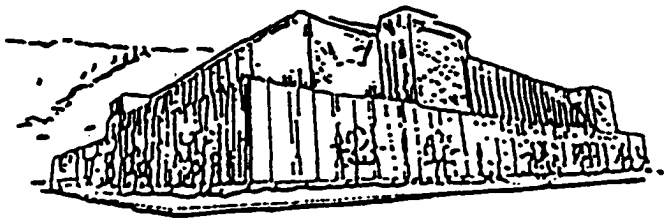

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Introduction to the *Bartonella* and Genetic Manipulation

I. THE BARTONELLA

A. INTRODUCTION

The bartonellae are a unique group of gram negative intracellular bacteria that employ arthropod-mediated transmission and hemotrophy as common parasitic strategies. Phylogenetically, recent taxonomic re-classifications have expanded the number of *Bartonella* species from one, *B. bacilliformis*, to 11 species, based on sequence homology and genetic relatedness. Five of these species are presently considered agents of emerging infectious disease in humans (*B. bacilliformis*, *B. clarridgeiae*, *B. elizabethae*, *B. henselae*, and *B. quintana*), and share pathologies of bacteremia, anemia, relapsing fever, and a variety of angiomatous lesions (for recent reviews see references 7, 91, and 125). Human morbidity and mortality have been associated with numerous manifestations of the *Bartonella*, and are explained in detail below.

A considerable amount of data have been accumulated concerning the epidemiology of the bartonellae. With the exception of *B. bacilliformis*, which is uniquely endemic to the Andes Mountains of South America, there are apparently no geographic boundaries for the remainder of the genus. Bartonellae have been isolated from a variety of sources including cats, dogs, birds, fish, rodents, and even plants. Arthropod-mediated transmission is the primary route of infection, and a diverse range of insect vectors including sandflies, mites, lice, fleas, and ticks have been shown to harbor bartonellae. The combination of reservoir and vector diversity, as well as reports of co-isolation of bartonellae with other human pathogens, has lead to much discussion of the epidemic potential of this genus. A

thorough summary of the hosts, reservoirs, and vectors is presented below.

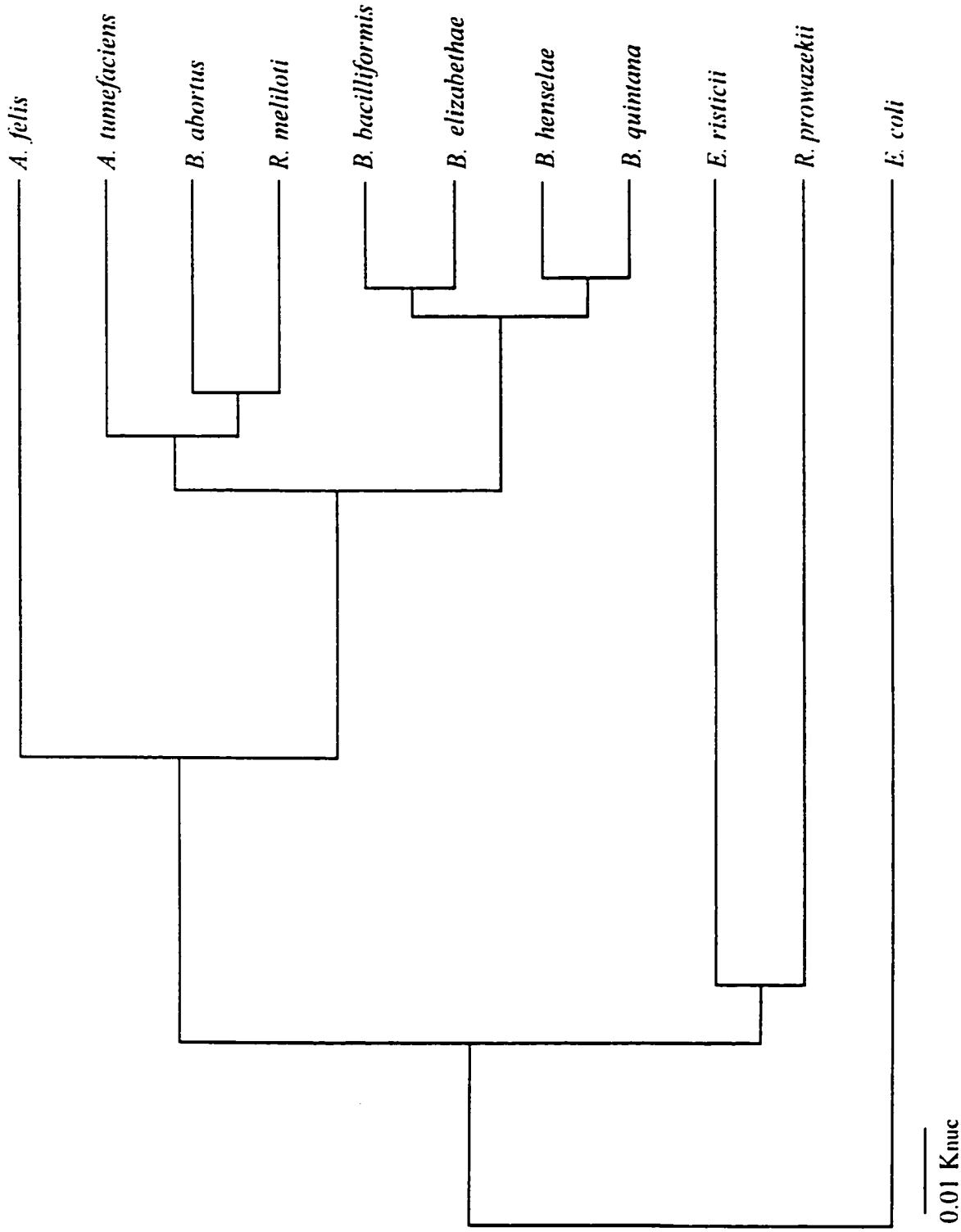
It is noteworthy that there is a dearth of information concerning the pathogenesis and epidemiology of the obscure pathogen *B. bacilliformis*. Probably due to higher levels of morbidity and mortality, much more has been published (in English) pertaining to *B. henselae* and *B. quintana*. These former members of the *Rochalimaea* share many characteristics with *B. bacilliformis* which will be mentioned below to provide a more comprehensive understanding to the reader.

B. PHYLOGENY OF THE BARTONELLA

The type species of the *Bartonella* genus, *B. bacilliformis*(26), was originally classified as belonging to the order Rickettsiales of the alpha *Proteobacteria* and was the only species in the family *Bartonellaceae*. Recent taxonomic reclassifications based on sequence homology, DNA hybridization data and G+C content have removed the family *Bartonellaceae* from the order *Rickettsiales* and placed it in the order *Rhizobiaceae* (27). In addition, members of the family *Rickettsiaceae* belonging to the genera *Rochalimaea* including *R. quintana*, *R. vinsonii*, *R. elizabethae*, and *R. henselae* were reassigned to the family *Bartonellaceae* and subsequently the genus *Rochalimaea* was added to the group (27). Finally, the genus *Grahamella*, which was a late addition to the family *Bartonellaceae*, was unified with the *Bartonella* genus (21). All of these taxonomic changes were proposed between 1993 and 1995, and have been corroborated by several sources (19, 22, 26, 33, 40, 105, 120, 126-128, 137, 148, 172) included in this is the high degree of sequence similarity between the *B. bacilliformis* flagellin sequence and other flagellins in the alpha *Proteobacteria* (135). Interestingly, the *Bartonellaceae* share homology with mitochondrial and unidentified symbiont sequences.

If these taxonomic rearrangements are reminiscent of a daytime drama, please refer to Figure 1, a phylogenetic tree derived from 16S DNA sequence alignments (Adapted

Figure 1. Phylogeny of the *Bartonella*. This phylogenetic tree was adopted from a previous publication by M.F. Minnick in (124). Construction was achieved using 16S rDNA sequences of the mentioned bacteria from GenBank, comparison of the sequences was accomplished using Clustal software (PCGene 6.0; Intelligenetics). K_{nuc} is a measure of evolutionary distance. The phylogenetic relationships of several α-proteobacteria as well as the prototypic γ-Proteobacteria, *Escherichia coli*. Notice the likeness between the plant pathogens (*Agrobacterium tumefaciens* and *Rhizobium meliloti*) and the *Bartonella*. Four of the eleven species of *Bartonella* are provided here. For a complete list refer to Figure 2. The species shown as listed from the top are *Afipia felis* (originally suspected as the etiologic agent of cat-scratch disease), *Agrobacterium tumefaciens*, *Brucella abortus*, *Rhizobium meliloti*, *Bartonella bacilliformis*, *B. elizabethae*, *B. henselae*, *B. quintana*, *Ehrlichia risticii*, *Rickettsia prowazekii*, and *E. coli*.



from (124). The dendrogram does not include several of the new *Bartonella* species, and the reader is referred to Table 2 below for a complete listing of all of the species in the *Bartonella* genus to date.

C. HISTORY AND EPIDEMIOLOGY OF THE *BARTONELLA*

1. *B. bacilliformis.*

The diseases caused by *B. bacilliformis* have been dated as early as pre-Columbian times by anthropomorphic pottery depicting individuals with lesions representative of verrugas (180); the cutaneous eruptions associated with the second phase of the disease. Recent anthropological data has identified verrugas on a pre-Columbian Peruvian mummy, apparently a human sacrifice, and used transmission electron microscopy (TEM) to show bacilli with polar flagella within the verruga representative of *B. bacilliformis* (3).

The cutaneous eruptions caused by *B. bacilliformis* and the eukaryotic pathogen *Leishmania viannia peruviana* (or *L. mexicana*) were referred to in folklore by the natives of South America as 'Uta', which was their name for the sandfly associated with both diseases (72). The native terminology for the diseases has become more specific since that time; 'Uta' has become synonymous with cutaneous leishmaniasis caused by *Leishmania viannia peruviana* (2), and *B. bacilliformis* maladies are referred to as mal de Carrión. Interestingly, these pathogens not only share similar clinical presentations (cutaneous eruptions), but both *L. viannia peruviana* and *B. bacilliformis* share the unique geographic endemicity of the Andes Mountain region of South America. It has been demonstrated that over 60% of the asymptomatic population in this region is seropositive for *B. bacilliformis* and 5-10% are active carriers of the infectious agent (89, 103). The primary reason for this limited distribution is believed to be the habitat of the phlebotamine sandfly *Lutzomyia verrucarum* that is the arthropod vector associated with both diseases.

TABLE 1: Significant Moments in the History of the *Bartonella*

Year	Event	Source(s)
1540	Pizarro's companions note 'verrugas' among the 'conquistadors'	(53)
1630	First account of human bartonellosis	(71)
1764	First account of sandfly vector	(71)
1858	First description of anemic phase by Salazar	(53)
1870	First recorded major epidemic; Peru, ~8000 fatalities	(178, 53)
1885	D. Carrión dies while demonstrating biphasic nature of the disease	(178)
1905	A.L. Barton first reports *B. bacilliformis*	(149, 178)
1915	Strong *et al.* confirm biphasic nature of the disease	(178)
1915	Millions of soldiers suffer from Trench fever caused by *B. quintana* *	(143)
1926	Noguchi and Battistini demonstrate Koch's postulates	(178)
1929	Noguchi and Shannon confirm Phlebotamine vector	(178)
1938	Second recorded major epidemic; Columbia, ~4000 fatalities	(178)
1959	Peruvian outbreak, ~200 deaths	(178)
1987	Peruvian outbreak, 14 deaths	(61)
1988	First report of bacillary angiomatosis; new *R. henselae* pathogenesis*	(104)
1993	Family *Bartonellaceae* placed in the order *Rhizobiaceae*	(27)
1993	*Rochalimaea* genera unified with genus *Bartonella*	(27)
1995	Genus *Grahamella* unified with genus *Bartonella*	(21)

* Indicates species of Bartonella other than *B. bacilliformis*.

In 1929, the folklore of the natives was verified when Battistini, Noguchi and Shannon identified *Lutzomyia* sandflies (formerly *Phlebotomus*) as the vector for *B. bacilliformis* (73, 180). *Lutzomyia* lives in narrow valleys and cooler areas primarily between the altitudes of 500-3000 meters above sea level in the Andes Mountain regions of Peru, Columbia, and Equador (103). Several studies have implicated the female of the species as the responsible vector, and that human infection takes place at night, when the environmental temperatures are between 18°C and 20°C (71, 73). Furthermore, studies have demonstrated that the bacteria produce a massive proboscis and pharynx infection in the sandfly (73) The male sandfly has not been associated with human blood meals (71, 73), but alternatively has been observed feeding on plants (personal communication, R. Birtles, 1997).

The question of the *B. bacilliformis* reservoir is still ambiguous. Native folklore has instigated several studies of euphorbiacous plants as a possible reservoir. In 1930, Maldonado noted a connection between the 'verruga zones', or endemic areas, and these euphorbiacous plants (71). In the same year, Weiss isolated organisms similar to *B. bacilliformis* from the latex of these plants, and shortly thereafter, Mackehenie isolated and cultured these organisms from the plant latex on three separate occasions and was able to produce verruga-like nodules in chickens (71). These data led to a proclamation in 1935 by Escomel to destroy the suspected plants, *Jatropha macrantha* and *Jatropha basiacantha* (71). In 1953, Herrer attempted to reproduce these results and also tried to grow *B. bacilliformis* on the plant latex, without success (71). The frequency of disease incidence is directly correlated with the rainy seasons (December-April), and is the time when these plants leaf, corroborating the plant reservoir hypothesis (71). Considering the close phylogenetic relationship that *B. bacilliformis* has with plant pathogens such as *Rhizobium* and *Agrobacterium* (181), and the observation that the male *Lutzomyia* feeds on plants (personal communication, R. Birtles, 1997), this hypothesis is firmly established. Finally,

sandflies have been observed feeding on pigs, field mice, dogs, monkeys, chickens doves, and plants, and interestingly are not attracted to lizards (72), (personal communication, R. Birtles, 1997). Thus far, humans are the only widely accepted reservoir for *B. bacilliformis* (70, 72).

With one unsubstantiated exception (182, 185), all documented cases of *B. bacilliformis* infection have been associated with these geographic and vector restrictions. New reports of an atypical manifestation of *B. bacilliformis*, presenting with cutaneous eruptions in the absence of the primary anemic phase, have been causing some concern (2, 4). Amano *et al.* describe these atypical monophasic presentations as emerging in previously disease-free lowland elevations (2, 4). The authors suggest that since *Lutzomyia verrucarum* has not been observed at this low altitude, a new strain of *B. bacilliformis* or a unique species of *Bartonella*, with an alternative vector, may be the cause.

To provide a more comprehensive summary of the epidemiology of the *Bartonella*, the remainder of this section will focus on other *Bartonella* species. Of the remaining *Bartonella* that are pathogenic to humans, *B. quintana* and *B. henselae* have been associated with the highest numbers of human morbidity and mortality.

2. *B. quintana.*

B. quintana, the first identified *Rochalimaea* infection (90), caused over one million cases of morbidity in military personnel during World War I (83, 144). In recent past there has been a resurgence of this disease in homeless persons, alcoholics and intravenous drug users in inner-city populations (29, 36, 44, 45, 83, 84, 142, 147, 167). In contrast to *B. bacilliformis*, *B. quintana* apparently has no geographic restrictions, and has been reported in France, England, Canada, Africa (29, 142), Seattle, Washington (84), and Baltimore, Maryland (36).

In several of these case studies, there was a direct correlation between disease

Table 2: The *Bartonella* Genus

Species	Human Disease	Vector	Source(s)
B. bacilliformis	Oroya Fever, Verruga peruana	Sandfly	(72, 79, 178)
B. quintana	Trench Fever, BA, endocarditis	Human body louse, fleas?	(175)
B. henselae	CSD, BA, endocarditis	Flea, Cat-scratch	(143, 93)
B. elizabethae	endocarditis	unknown	(37)
B. clarridgeiae	CSD	cat scratch	(94)
Species not yet associated with human disease			
B. vinsonii			(184)
B. talpae, *B.peromysci* , *B. grahamii*, *B. taylorii*, and *B. doshiae*			(21)

frequency and alcoholism or drug use. The rate of infection, as well as the variety of clinical pathologies reported in immunocompromised individuals, is significantly higher than immunocompetent individuals, although infection does occur in immunocompetent individuals (10, 35). *B. bacilliformis* and *B. quintana* share two epidemiological features; a single known reservoir, and a single reported vector. First, humans have been identified as the only reservoir for *B. quintana* , and second, this species has been associated only with a single vector, *Pediculus humanus humanus*, the human body louse (82, 83, 177) as the sole vector for this species.

3. *B. henselae.*

To date, *B. bacilliformis* has caused more deaths than any other *Bartonella*, and *B. quintana* is responsible for the highest numbers of human morbidity. Recently, however, *B. henselae* has emerged as the predominant species causing a variety of clinical pathologies in both immunocompetent and immunosupressed individuals. This organism was originally identified in HIV-1 individuals presenting with cutaneous lesions similar to, but distinct from, Kaposi's sarcoma (34, 40, 105), termed bacillary angiomatosis (BA) (146). Shortly thereafter, *B. henselae* was recognized as the etiologic agent of cat-scratch disease (CSD) (8, 27, 144, 184). Although *Afipia felis* was originally thought to be the etiologic agent of CSD, *B. henselae* is now accepted as the cause. In general, *B. henselae* infection of healthy individuals results in CSD, whereas infection of the immunocomprimised results in BA. Both syndromes are discussed in section E, below.

The epidemiology of *B. henselae* is unique in that the domestic cat, *Felis domesticus*, has been positively identified as a relatively asymptomatic reservoir (70, 87, 111, 172, 174). There is apparently no geographic restrictions of endemicity, for the bacterium has been observed in cats throughout the world (32, 70, 87, 99, 111, 190). Approximately one third of all United States households own at least one cat,

corresponding to over 57 million pet cats in the U.S. alone (94).

The cat flea, *Ctenocephalides felis* (94), has been established as the arthropod vector for *B. henselae* by experimental transmission from infected cats to specific pathogen free cats (32), and by injection of live suspensions (64). Clinical symptoms of experimental infection in the cat include fever, lethargy, and lymphadenopathy, which dissipate quickly and a bacteremic state subsequently ensues for up to 32 weeks (64). Interestingly, in the U.S. there is a direct correlation between cat flea prevalence, yearly average temperature, and percent of seropositive cats (87). Finally, although *B. henselae* has not been detected in dogs, *Ctenocephalides felis* has also been shown to infest dogs (172).

4. Other *Bartonella*.

Very little is known regarding the epidemiology of the two remaining human *Bartonella* pathogens, *B. clarridgeiae*, and *B. elizabethae*. *B. clarridgeiae* has been isolated from cats independently (70), or as a co-infecting agent with *B. henselae* (65). *B. elizabethae* has only been associated with human endocarditis (38) and CSD (33).

The other six described species of bartonellae have their roots in the former *Grahamella* genera (20). Although not associated with human morbidity or mortality, they have been observed in rodents, fish, mammals and birds (20), and are the cause of pathologies in other mammals that resemble human maladies (25, 96, 186). There is also a wide range of arthropod vectors associated with these remaining species including mites (12), fleas (103) and ticks (77).

Finally, it is notable that different *Bartonella* species have been found together in nature (65, 77). A recent report that emphasizes this notion was the co-segregation of a novel *Bartonella* species with both *Borrelia burgdorferi* and the hemotrophic parasite *Babesia microti* (77). The diversity of reservoirs and vectors of the *Bartonella* combined

with the notion of virulence-gene transfer with different *Bartonella* species as well as with alternate pathogenic bacteria (*B. burgdorferi*) may extend the emergence of the *Bartonella* for many years to come.

D. PHYSICAL CHARACTERISTICS

The *Bartonellae* are small, gram negative, aerobic, pleiomorphic bacteria varying between coccoid and bacilliform cellular morphologies. *B. bacilliformis* has a size of approximately 1.3-3.0 μm long by 0.25-0.5 μm wide (26, 103, 175, 181). These microorganisms are fastidious, facultative intracellular pathogens that are generally cultivated between 30°C and 37°C in the presence of hemin or erythrocyte lysate, and serum (118), and are oxidase negative, catalase positive, and non-fermentative (26). *B. bacilliformis* exhibits two different colony morphologies, termed T1 and T2, that have been shown to have differential degrees of erythrocyte adherence and interconvert upon passage(179).

Both *B. bacilliformis* (180) and *B. clarridgeiae* (33, 95) constitutively express a tuft of 1-10 polar flagella filaments from one pole, which is termed a lophotrichous flagella. *B. henselae* has also been shown to express flagella under certain growth conditions (personal communication, K. Karem, 1997). The flagellum provides *B. bacilliformis* with a high degree of motility (26, 115, 116, 164, 179), and is the virulence determinant that we focused on in this dissertation. The 42 kDa (164) flagellin subunit of *B. bacilliformis* has been identified as highly immunogenic (124). One report identifies 14 outer membrane proteins (OMP's) which range in size from 11.2 kDa to 75.3 kDa, and designates three of these OMP's, 31.5 kDa, 42 kDa and 45 kDa as highly immunogenic (124). Finally, the LPS of *B. bacilliformis* has been shown to be a poor immunogen (124) but it does induce hyper-reactivity in rabbits (130).

The *Bartonellae* have an AT-rich genome; the type strain of *B. bacilliformis* has a G+C content of approximately 40% (26), and *B. quintana* has been shown to have 38.6% G+C (173). The *B. bacilliformis* chromosome is a single circular molecule of approximately 1,600 kbp (104). Bacteriophage-like particles (BLP) have been identified in both *B. bacilliformis* (175) and *B. henselae*(5). The BLP's evidently package random 14-kbp segments of double-stranded DNA into their acid-resistant protein coats (5, 24)(personal communication, M.F. Minnick 1998). Neither *B. quintana*, *B. elizabethae*, nor *B. vinsonii* has demonstrated this 14 kbp extrachromosomal segment, and is thus thought to be lacking BLP's (5). It is interesting that a major protein of this BLP coat, Pap31, shares 68% sequence similarity with an outer membrane protein of *Rhizobium leguminosa*, a plant-associated bacterium (24). In addition to BLP's, *B. quintana B. henselae*, and *B. bacilliformis* have been shown to form membrane blebs of unknown function (28)(personal communication, M.F. Minnick 1998).

E. PATHOGENESIS

In general, the *Bartonella* are the only hemotrophic bacteria infectious to humans (153). Hemotrophy is the parasitism of erythrocytes. There are several human hemotrophic maladies which arise from protozoa (plasmodia, babesia, theileria, and trypanosomiasis) (103), and several hemotrophic bacteria which have been isolated from other mammals (Eperythrozoa, Anaplasmataceae, haemobartonellae) (43, 103, 153). It is interesting that all hemotrophic bacteria identified thus far are arthropod borne, and have a variety of vectors including ticks, sandflies, fleas, and lice (103).

Most of the diseases caused by *Bartonella* have three common themes; hemotrophy, infection of endothelial tissue, and immunosuppression. The hemotrophy can range from acute bacteremia to the severe hemolytic anemia as manifest in the primary phase of *B. bacilliformis* infection. Several sequelae result from cutaneous or sub-

cutaneous endothelial cell invasion by the bartonellae in immunocompetent individuals. These include the maculopapular rash associated with classical *B. quintana* infection, the cutaneous granulomatous lesions of cat-scratch disease resulting from *B. henselae*, and the angiomatous eruptions caused by *B. bacilliformis*. In general, infections in immunosupressed individuals can result in a variety of atypical consequences in which the bartonellae can invade endothelial cells throughout the body causing angiomatous lesions from the liver to the brain. The bartonellae are the only infectious agents reported to cause angiomatous lesions (37). In fact, immunosuppression appears to be induced by these organisms and often results in secondary opportunistic infections that complicate diagnosis and treatment.

Clinical manifestations in both immunocompetent and immunosupressed individuals are explained in detail below. For further reading, refer to two excellent reviews of the pathogenesis of *Bartonella* infections by Minnick (125) and Koehler (91).

1. Carrión's disease: Oroya Fever and Verruga peruana

B. bacilliformis is the etiologic agent of a biphasic disease in which the primary (or hematic) phase is associated with fever and anemia, and the second (or tissue) phase is one in which the organism parasitizes endothelial cells resulting in vascular lesions. The term Carrión's disease is used in reference to both the primary phase as well as the entire biphasic syndrome. Oroya fever is commonly used to describe the first phase, and is characterized by an acute syndrome of fever, malaise, and a severe febrile hemolytic anemia (54, 150, 180). Humans present with this acute hematic phase of the disease within 2-3 weeks following inoculation of bartonellae into the bloodstream by the bite of a nocturnal sandfly, *Lutzomyia verrucarum* (62). During this hematic phase the bacterium invades nearly every erythrocyte in the blood and subsequently reduces the hematocrit by nearly 80% (81). The anemia of this phase of the disease is responsible for the high (40-80%)

mortality rate in the absence of antibiotic therapy (62, 103), and has killed over 10,000 humans in recorded time (62, 180), and affected many others (62, 108, 112). Reports have stated that this anemia is a result of the reticuloendothelial system (RES) attempting to eliminate the bacteria by phagocytosis (143, 150). Furthermore, Dealler, *et al.* state that *B. bacilliformis* induces a severe immunosuppression and that this is the cause of most of the deaths (40). Bacteremia is often a manifestation of infection with several of the *Bartonella*, as will be explained below.

The chronic secondary phase of the disease, termed verruga peruana (Peruvian warts), develops weeks to months after infection and is characterized by blood-filled cutaneous eruptions on the extremities (62). During this phase, *B. bacilliformis* invades vascular endothelial cells, the epithelium lining the blood vessels (53, 54, 115), and presents with hemangiomas on the face and extremities, immunosuppression, hepatosplenomegaly and lymphadenopathy (54). A novel approach to the pathogenesis of this phase was proposed by Recavarren and Lumbreras in which the human body drives the bacteria to the skin, and the verrucous tissue develops to eliminate the *Bartonella* during this second phase (143). The majority of data support the contrasting notion, in which the bacteria induce the angiomatous lesions. For example, Garcia *et al.* demonstrated that *B. bacilliformis* possess a factor larger than 12-14 kDa which induces the release of tissue plasminogen activator (t-PA), an angiogenic metabolite, from endothelial cells (52). Furthermore, *B. bacilliformis* extracts were shown to stimulate formation of new blood vessels in *in vivo* experiments with rats (52). Endothelial cell invasion is a common pathogenic technique used by a number of the *Bartonellae*.

2. Trench Fever

Bartonella (formerly *Rochalimaea*) *quintana* caused morbidity in millions of soldiers suffering during World War I (83, 144, 147) and has been reemerging primarily in inner

city homeless populations during the last 15 years. (29, 36, 44, 45, 83, 84, 142, 147, 167). The classical disease caused by *B. quintana*, called Trench Fever, Shinbone Fever, or Five Day Fever presents with relapsing fever, malaise, bone pain, chills, headache and a maculopapular rash (177) after inoculation by the human body louse *Pediculus humanus humanus* (82, 83). In immunocompetent individuals this disease is usually self-resolving, and symptoms can even be so mild that they are not recognized.

Although it was originally reported that *B. quintana* did not invade endothelium in either the louse gut (82) or human tissue (13) , it has been recently demonstrated that *B. quintana* does invade endothelium, consistent with the other *Bartonella* (28). *B. quintana* has been associated with a number of unusual clinical presentations in both immunocompetent and immunosupressed individuals including endocarditis and a variety of bacillary angiomatosis sequelae. These maladies will be discussed according to specific disease below.

3. Cat Scratch Disease (CSD)

Next to Trench Fever, Cat-scratch disease is the *Bartonella* infection which has gained the most notoriety in the recent past (likely due to Ted Neugent's rendition of "Cat Scratch Fever"). Cat-scratch disease is a result of either *B. henselae* infection (15, 42, 69, 190) or *B. clarridgeiae* infection (33). The disease states range from an asymptomatic or a self-resolving cutaneous granulomatous lesion (15, 42, 69, 190), to a variety of serious sequelae such as endocarditis and bacillary angiomatosis (below). The latter of the two disease states usually occurs in children and immunosupressed individuals as a result of *B. henselae* (or *B. quintana*) infection and subsequent bacteremia. Both endocarditis and bacillary angiomatosis are explained below.

4. Endocarditis

Endocarditis is characterized by various alterations of the endocardium including exudative, ulcerative, proliferative, and vegetative lesions and is most often associated with a heart valve. A variety of infectious agents are known to cause endocarditis including *Staphylococcus*, *Sreptococcus*, *Rickettsia*, *Mycobacterium*, and various mycotic agents, which makes diagnosis a challenge. *Bartonella* endocarditis is most often associated with *B. henselae* infection (46, 79, 142), but has also been demonstrated resulting from *B. elizabethae* (38), and *B. quintana* (44, 86, 142). This disease most often presents in alcoholics (86) or immunosupressed individuals, but has also been demonstrated in immunocompetent adults (79). In addition, a report of dog endocarditis was diagnosed as *B. vinsonii* (25).

5. Bacillary Angiomatosis (BA)

Angiogenesis is a complicated process that results in the formation of new blood vessels (90), and is often associated with cancerous tumors. Cutaneous presentation of bacillary angiomatosis (BA) clinically resembles Kaposi's sarcoma and was thus associated with this early manifestation of HIV-1 infection (34). It was noticed at this time that these epithelioid vascular proliferations clinically resemble the cutaneous eruptions called verruga peruana of *B. bacilliformis* infection (40, 105). Further investigation revealed histological evidence that BA was distinct from Kaposi's sarcoma having an epithelioid hemangioma appearance (34).

Bacillary angiomatosis, a 'bacilliform-induced angiomatous lesion', is characterized by nodular, cutaneous, visceral, or lymphatic lesions and has vascular channels lined with cuboidal epithelium (37, 144, 146). It was demonstrated that endothelial cell proliferation and migration are remarkably stimulated in the presence of *B. henselae* and *B. quintana*, and that fibroblast cells are unaffected (37). Furthermore, a trypsin-sensitive factor which

is either a cell wall component or a very heavy intracellular molecule was identified with this proliferation and migration activity (37).

The infection of *B. quintana* and *B. henselae* has only been reported to occur from cat-scratches or bites, flea to human transmission has not been documented, but has been hypothesized (94). After infection, the sequelae in immunocompetent hosts is usually asymptomatic or CSD ensues and is self-resolving. In children and immunosupressed adults, a variety of maladies can occur, ranging from cutaneous BA (10, 34, 105), to bacteremia and migration throughout the body. Reports of bacteremia (144, 166), granulomatous hepatitis (106), peliosis hepatis (55, 141), osseous lesions (93), encephalitis (169), status epilepticus and coma (67), psychiatric symptoms (11), pulmonary nodules (30), intra-abdominal mass and gastrointestinal hemorrhage (92), pyogenic granuloma (10), polyadenopathy and splenomegaly (10), neuroretinitis (188), aseptic meningitis (188), lymphadenitis (188), brain lesions and CNS infection (140), seizure (154), prolonged fever and fever of unknown origin (FUO) (85), and hypercalcemia (23) have been associated with either *B. henselae* or *B. quintana*.

F. VIRULENCE DETERMINANTS

The pathogenicity of an organism is defined as it's ability to cause disease. The virulence determinants, also called virulence factors, are the tools or mechanisms that an organism uses in pathogenesis. To be pathogenic, *Bartonellae* must first be able to survive the extreme disparate environments of arthropod vector and reservoir. Second, either by a specifically designed mechanism or a serendipitous circumstance of natural existence, the bacteria then manifest their pathogenicity on the host. Methods of colonization, nutrient acquisition, temperature acclimation, serum resistance, immune evasion, erythrocyte and endothelial cell invasion, and angiogenesis are presently included in the pathogenesis of these bacteria.

The determinants utilized by *Bartonella* during pathogenesis, or virulence factors, are poorly understood. Much of the research to date has focused on mechanisms of host cell interactions with erythrocytes (14, 132, 164) and endothelial cells (28, 37, 52, 53, 90). Reports concerning adhesion and invasion implicate three main virulence determinants; the flagella (14, 164), the deformin protein (116, 189), and the IalA and IalB proteins encoded by the invasion-associated locus, *ialAB* (132). In addition, reports of a hemolysin (129), heme-binding protein (personal communication, M.F. Minnick, 1998), putative fimbriae (121), bundle-forming pili (BFP) (114), heat shock protein (122), an angiogenic factor (37, 52), and a unique blebbing surface appendage (28) are included in the possible virulence factors that are involved in host cell invasion.

Despite these studies, the mechanism by which *Bartonella* gain entry into erythrocytes is unknown. The working model of *B. bacilliformis* erythrocyte invasion is termed 'forced endocytosis'. Interaction and adhesion with the erythrocyte surface may be influenced by the following structures: flagella (164), filament A (121), and BFP (114). Subsequently, the activity of three virulence factors, IalA/IalB (132), deformin (116, 189), and the flagella (14, 164), propagate erythrocyte invasiveness by separate mechanisms and are explained below.

Conversely, invasion of endothelial cells may involve induced endocytosis. Erythrocytes are not capable of endocytosis (116) whereas endothelial cells can be stimulated to (de)polymerize actin and perform endocytosis. Thus, endothelial cell invasion may utilize a separate mechanism. One finding that supports this notion is a bleb-associated surface appendage (28) that was reported to induce rapid endothelial cell engulfment of *B. quintana* (invasion within one minute). This report contradicts early data concerning *B. quintana* invasion, where it was previously thought to remain epicellular (13, 98). Although blebs have been seen in association with *B. bacilliformis*, their relationship to surface appendages and endothelial cell engulfment is unknown.

Interactions of *B. bacilliformis* with endothelial cells are followed by a rapid invasion (within one hour) (53), a reduction of invasion with endothelial cells preexposed to cytochalasin D (115) suggests that microfilament formation is required for invasion of endothelial cells. These data suggest that the *Bartonella* use a mechanism of induced phagocytosis to enter endothelial cells.

1. Adhesion to Host Cell: Flagella, BFP, filA, and a blebbing surface appendage.

After the bacterium is introduced into the host following arthropod inoculation or cat-scratch, it is hypothesized to form a close contact, or adhere, to erythrocytes and/or endothelial cells. Three membrane localized appendages have been implicated in the process of *B. bacilliformis* adherence to erythrocytes; the flagella (164, 179), bundle-forming pili (BFP) (13, 114), and filament A protein (121).

In addition, adherence of *B. bacilliformis* is reduced after treatment of the erythrocyte with α- or β-glucosidase (catalyzes hydrolysis of terminal β-glucose residues from glycosides), and is stimulated after treatment of the erythrocyte with pronase or subtilisin (proteolytic enzymes)(179). These observations suggest that *B. bacilliformis* adheres to a glycolipid moiety on the erythrocyte surface (179).

a. The Flagella.

The data presented in chapter three suggest that the *B. bacilliformis* flagella is required for full host cell adherence capacity. Thus, the flagella may provide a motive force and thereby permit other surface appendages (such as fimbriae or pili) to form a more intimate bond with the host cell surface receptor(s). Conversely, the flagellum itself could be involved with the host cell receptor directly. The data reported by Scherer *et al.* also

suggest that the flagella may be involved in adhesion to the erythrocyte surface, for association rates are decreased in the presence of anti-flagellin antiserum (164).

b. BFP

Fimbriae and pili are cell surface appendages and are virulence factors associated with cellular adherence and colonization by a number of pathogenic bacteria. *B. henselae* possess an appendage that phenotypically resembles type 4 pili (13). Also characteristic of bacteria that possess type 4 pili, *B. bacilliformis* and *B. henselae* have demonstrated autoagglutination, in which the bacteria clump in groups of 5 to 100's (13, 114). When subjected to specific purification strategies, the pili autoaggregate to form bundles, and are hence called bundle forming pili (BFP), ranging from 50 to 600 nm in diameter (114). These BFP were found to be resistant to treatment with 0.1% SDS and do not migrate into SDS-PAGE gels (114). To date, this putative adhesion virulence determinant is uncharacterized.

c. FilA

Recently, a gene located upstream of the *ialA/ialB* locus (described in 3, below) was sequenced and shown to have a 1,200-bp open reading frame that shares sequence homology with several filamentous proteins including the M1 protein of *Streptococcus pyogenes* (121). The M1 protein has been demonstrated to be involved in the adherence to and invasion of host cells of *Streptococcus pyogenes* (121). Due to it's filamentous nature, the gene was named *filA* and encodes a protein, FilA, with a predicted molecular mass of approximately 47 kDa (121). Further sequence analysis indicates that the predicted protein has a typical secretory sequence, a potential hydrophobic anchor, and a leucine-rich composition (121). These data coupled with the similarity to M1 protein suggest that this predicted protein may be surface localized and may be involved in host cell attachment.

Further analysis of this putative adhesin has been hampered by the inability to clone the gene in *E. coli* presumably due to toxic effects of the gene (personal communication, M.F.Minnick, 1998).

d. Blebbing surface appendage.

As was stated above, a blebbing surface appendage similar to those described for *Salmonella typhimurium* (57) and *Rickettsia conorii* (170) has been described for *B. quintana* and *B. henselae* (28). The blebs were apparently formed by cell wall modifications and were only expressed by bacteria that had not achieved adherence (28). Bacteria that had made contact with the endothelial cell surface induced host cell membrane ruffles and engulfment were observed within one minute of cellular contact (28). These tubular or vesicle-like structures are apparently involved in endothelial cell invasion of *B. quintana* and *B. henselae*, and thus may also be utilized by *B. bacilliformis*. The finding of reduced *B. bacilliformis* invasion of endothelial cells pretreated with cytochalasin D (115) supports the hypothesis of microfilament-assisted invasion, or 'induced phagocytosis'.

2. Invasion of Host Cell: Flagella, *ialA/B*, and Deformin

a. The Flagella.

The lophotrichous flagella of *B. bacilliformis* and the high degree of motility that it imparts has been implicated as a virulence determinant in several reports (14, 103, 116, 164). In addition, *B. clarridgeiae*, an etiologic agent of cat scratch disease, has also been reported as possessing a lophotrichous flagella (95). A dendrogram analysis of several flagellin sequences demonstrated that the *B. bacilliformis* Fla is most similar to flagella from *Azospirillum brasilense*, *Rhizobium meliloti*, *Agrobacterium tumefaciens*, and *Caulobacter crescentus*, forming a cluster distinct from other flagellins (135). The mutation data generated in this study suggest that the *B. bacilliformis* flagellar filament is composed

of multiple polypeptide subunits of 42 kDa encoded by a single flagellin gene, *fla* (chapter three), whereas many bacteria possess multi-subunit flagella .

Flagellum-mediated motility is a common strategy used by many pathogenic bacteria to colonize or gain access to host tissues or cells (139). Aflagellated or motility-impaired strains have decreased pathogenicity in numerous bacteria because of impaired colonization, adherence, or invasion, or by an uncharacterized method. Examples include *Borrelia burgdorferi* (161), *Campylobacter jejuni* (60), *Proteus mirabilis* (134), *Serpulina hyodysenteriae*(158), *Agrobacterium tumefaciens* (31), *Vibrio anguillarum* (138), *Vibrio cholerae* (152), *Salmonella typhi* (63), and *Helicobacter pylori* (48). Several reports have implicated uncharacterized motility-impaired *B. bacilliformis* as having a reduced pathogenicity (14, 116, 179). Namarski optics demonstrated that *B. bacilliformis* use a boring or twisting motion that is accompanied by a remarkable deformation of the erythrocyte surface (14). Scherer *et al.* were able to demonstrate that both erythrocyte invasion and association were decreased by treating the pathogen with anti-flagellin antiserum (164). Motile cultures of *B. bacilliformis* have been shown to have a higher rate of attachment and entry into erythrocytes (14, 116). Lastly, inactivation of proton motive force (N-ethylmaleimide) or respiration (KCN) significantly reduces binding of the bacterium to erythrocytes indicating that adhesion is energy-dependent (179). The flagella of *B. bacilliformis* is undoubtedly driven by a proton motive force (58, 110).

These findings strongly suggest that a forced endocytosis entry into erythrocytes requires flagellar-mediated motility. The data presented in chapter four suggests that the flagellum is required for adhesion to human erythrocytes. Molecular Koch's postulates (49) were applied to the flagella of *B. bacilliformis* by first constructing the flagellin minus mutant and a cognate complemented strain. The mutants were constructed by site-directed mutagenesis using a suicide plasmid, and the transcomplementation was accomplished by introduction of the wild-type flagellin gene on a replicative plasmid using

electrotransformation. These strains represent the first site-specific mutant and first transcomplement of any *Bartonella*. These data and a phenotypic analysis of the mutant and transcomplemented strains are presented in Chapter Three. A virulence assay was then developed and used to determine the virulence potential of the flagellin gene. In the data presented in chapter three of this work we demonstrate that the flagellum is required for wild-type rates of adhesion to human erythrocytes. This experiment is the first demonstration of molecular Koch's postulates for any of the *Bartonella*.

b. Invasion Associated Locus: IalA and IalB.

Attempts to identify determinants of virulence can be accomplished in several ways. In an elegant set of experiments performed by Mitchell and Minnick, the first virulence related gene of any *Bartonella* was identified (132). In this study, a 1,469-bp *Bam*HI fragment of *B. bacilliformis* chromosomal DNA was found to confer an erythrocyte invasive phenotype on minimally invasive strains of *E. coli* (132). Further cloning and sequence analysis identified a two-gene 'invasion associated locus' (ial) that was responsible for the invasive phenotype. Both of the genes, termed *ialA* and *ialB*, were found to be necessary for the invasive phenotype in *E. coli* (132). Recently, IalB was localized to the surface of *B. bacilliformis* by indirect immunofluorescence microscopy (personal communication, S. Coleman, 1998). Finally, *ialB* was site-specifically mutagenized utilizing the system developed and described in this dissertation (personal communication, S. Coleman, 1998). Virulence data concerning this second site-directed *B. bacilliformis* mutant are currently in progress.

c. Deformin

An extracellular protein termed deformin is generated by *B. bacilliformis* grown in a tryptone based medium and produces deep invaginations within erythrocyte membranes

(116, 189). Deformin consists of a dimer having an apparent native molecular weight of 130 kDa (116). These trenches and invaginations are more apparent in trypsin-treated erythrocytes, and the activity can be inactivated by protease treatment or a brief heat exposure of 70°C (116). Interestingly, pretreatment of the erythrocytes with phospholipase D produces deformin-resistant cells. This finding is consistent with the reduced adherence of *B. bacilliformis* after treatment of the erythrocyte with α- or β-glucosidase (179).

Scanning electron micrographs of deformin-treated erythrocytes show marked trenches, indentations and invaginations on the surface of the cells (189). By using Nomarski optics, *B. bacilliformis* was observed swimming into these induced trenches with a corkscrew-like motion and were able to gain entry into the erythrocyte and that non-motile bacteria never gained entry (14, 116). The data concerning this virulence determinant strongly support the current model of forced endocytosis.

3. Angiogenic Factor:

To date, the *Bartonella* are the only infectious agents associated with angiomatous lesions (37). As explained above, a factor is present in *B. bacilliformis* extracts that induces the release of tissue plasminogen activator (t-PA) from endothelial cells and is able to stimulate cellular proliferation of endothelial cells (52). This heat-sensitive factor is larger then 14 kDa and was shown to stimulate formation of new blood vessels *in vivo* (52). In another study, extracts of *B. henselae* and *B. bacilliformis* contained a trypsin-sensitive factor that induced the proliferation and migration of endothelial cells, but not fibroblast cells (37). This factor was established as either a cell wall component or a very heavy intracellular molecule and, interestingly, activity was not apparent in *B. quintana* extracts. These data support a pathogenic mechanism for the bacteria analogous to tumor formation, whereby an endothelial cell site is colonized and subsequently direct

nutrients to itself by the formation of new blood vessels.

The induction of t-PA release in endothelial cells by a *Bartonella* factor can have an alternative interpretation. t-PA converts plasminogen to its activated form, plasmin. Plasmin is a peptidase that dissolves thrombin, or clots, in the bloodstream. Streptokinase is a plasmin analogue produced by β-hemolytic streptococcus and is utilized by the organism as a spreading factor during infection. These data taken together suggest that the factor released by *B. bacilliformis* may indirectly reduce intravascular clotting (and thus keep the host alive), as well as act as a spreading factor during infection.

4. Immune Evasion Factors and Strategies.

The hemotrophic propensity of the *Bartonella* require that they have a mechanism for dealing with immune recognition and destruction. Two *in vitro* experiments testing humoral immunity demonstrated species-specific results. First, it was shown that human non-immune sera killed more than 99.5% of 10^7 *B. henselae* after 2 hours, by classical and alternate forms of complement fixation as well as phagocytosis (157). In contrast, experiments with *B. quintana* demonstrate that it is complement-resistant (117). This disparity between the humoral immunity of *Bartonella* species as well as the factor(s) involved still remains to be explained.

An obvious mechanism of immune evasion is intracellular invasion and survival. This also includes the rapid induced phagocytosis demonstrated by *B. quintana* (28). In this *in vitro* study it was shown that the bacterium is engulfed by the endothelial cell in less than one minute. The recurring 'five day' fever associated with *B. quintana* infection suggests that cell surface modulation may play a part in immune evasion. Finally, the blebs may be involved in immune avoidance. It was mentioned by Brouqui and Raoult that Borrelia uses blebs as a form of immune evasion, and that *Bartonella* may do the same

(28).

5. Stress Response Factors

Bacterial stress response factors include a group of proteins referred to as GroEL, GroES, DnaK, and HtrA (6). The temperature extremes of host (37°C) and vector (25°C) require that the *Bartonella* retain mechanisms for survival in these environments. *B. henselae* and *B. quintana* have at least five heat shock related proteins ranging from 10 kDa to 70 kDa (66). Anderson *et al.* have putatively identified a 62 kDa immunogenic protein in *B. henselae* that has sequence similarity to *E. coli* HtrA (6). The HtrA protein is required by *E. coli* for growth at elevated temperatures. Finally, Minnick demonstrated by single dimension SDS-PAGE that *B. bacilliformis* contains seven heat shock proteins ranging in size from 17.6 kDa to 66.5 kDa when heated to 37°C for 40 minutes (122). When applied to a two dimensional SDS-PAGE the same preparation revealed 23 heat shock proteins (122). It is interesting that *in vitro* growth of *B. bacilliformis* is most rapid at 30°C, whereas the bacteria barely survive even short exposures *in vitro* at 37°C.

6. Hemin Binding Factor:

The *Bartonella* are fastidious organisms that require a red blood cell lysate or hemin supplemented medium for survival and growth *in vitro* (118). In addition, their hemotrophic lifestyle suggests that a factor(s) may be involved in sequestration of hemin. To begin to answer this question, a *B. quintana* genomic library was made in the low-copy plasmid pACYC (personal communication, M.F. Minnick, 1998). Transformation of this library into *E. coli*, followed by selection on LB+0.5% hemin isolated several reddish-brown recombinants. Interestingly, sequence analysis of the inserts of one of these recombinants suggests it is homologous to the virulence locus described previously for *B. bacilliformis* (118, 121, 131-133) (personal communication, M.F. Minnick, 1998).

G. DIAGNOSIS, TREATMENT, and PREVENTION

1. Diagnosis.

The lesions produced by *B. bacilliformis* (verruga peruana), *B. henselae* (BA), and *B. quintana* are clinically indistinguishable from each other yet form unique cutaneous angiomatous eruptions. Due to the varied presentations of bacillary angiomatosis, clinical diagnosis can often be confused with salmonellosis, toxoplasmosis, tularemia, brucellosis, histoplasmosis, Q-fever, coccidiomycosis, amoebiasis, Epstein-Barr virus, tuberculosis, tetanus, syphilis, toxocariasis, rocky mountain spotted fever, chlamydia, and leptospirosis (106), ehrlichiosis (166), malaria (155), UTI and osteomyelitis (85). In addition, *Bartonella* infections that produce endocarditis can be confused with *Staphylococcus*, *Streptococcus*, *Rickettsia*, *Mycobacterium*, and various mycotic agents. To further complicate diagnosis, *B. quintana* infection was produced in a patient in the absence of seroreactivity (45).

These inherent diagnosis complications as well as the slow and fastidious nature of *in vitro* culture provide the impetus towards the development of a specific method for clinical identification. PCR techniques using amplimers directed to unique targets have been used successfully to diagnose *Bartonella* infections (8, 86, 88, 108, 136, 156), and differentiate between *Bartonella* species (123, 160).

2. Treatment.

Although the bartonellae are highly susceptible to even low levels of antibiotics (113), intracellular survival and opportunistic infections often require rigorous antimicrobial treatment. A variety of antimicrobial agents have been used successfully to treat different *Bartonella* infections including chloramphenicol (2, 180), doxycycline (30), erythromycin (141), gentamicin (45), β-lactams (180), streptomycin plus tetracycline (2,

180), and aminoglycosides (180). Finally, enrofloxacin and doxycycline was successfully used for treatment of *B. henselae* or *B. clarridgeiae* in cats (97).

3. Prevention.

In Chapter Three of this work we satisfy molecular Koch's postulates with the flagella of *B. bacilliformis*. This is the first demonstration of Koch's molecular postulates for any of the *Bartonella* or Rickettsial organisms. The data indicate that the flagellum is required for adhesion to erythrocytes. This suggests that a subunit vaccine incorporating flagellin could be an effective means of vaccination. Variable expression of the flagella is of course an argument concerning this notion. Overall, the significance of the development of this system of genetic manipulation relates directly to determination of virulence mechanisms and thus to vaccine production.

A vaccine has been made to protect cats from *Bartonella* infection. A *B. henselae* vaccine for cats has been produce by Heska Corporation (personal communication, K. Karem, 1997) developed for pet kittens of AIDS patients. No data is available on the efficacy of this vaccine.

II. GENETIC MANIPULATION

A. Introduction and Background

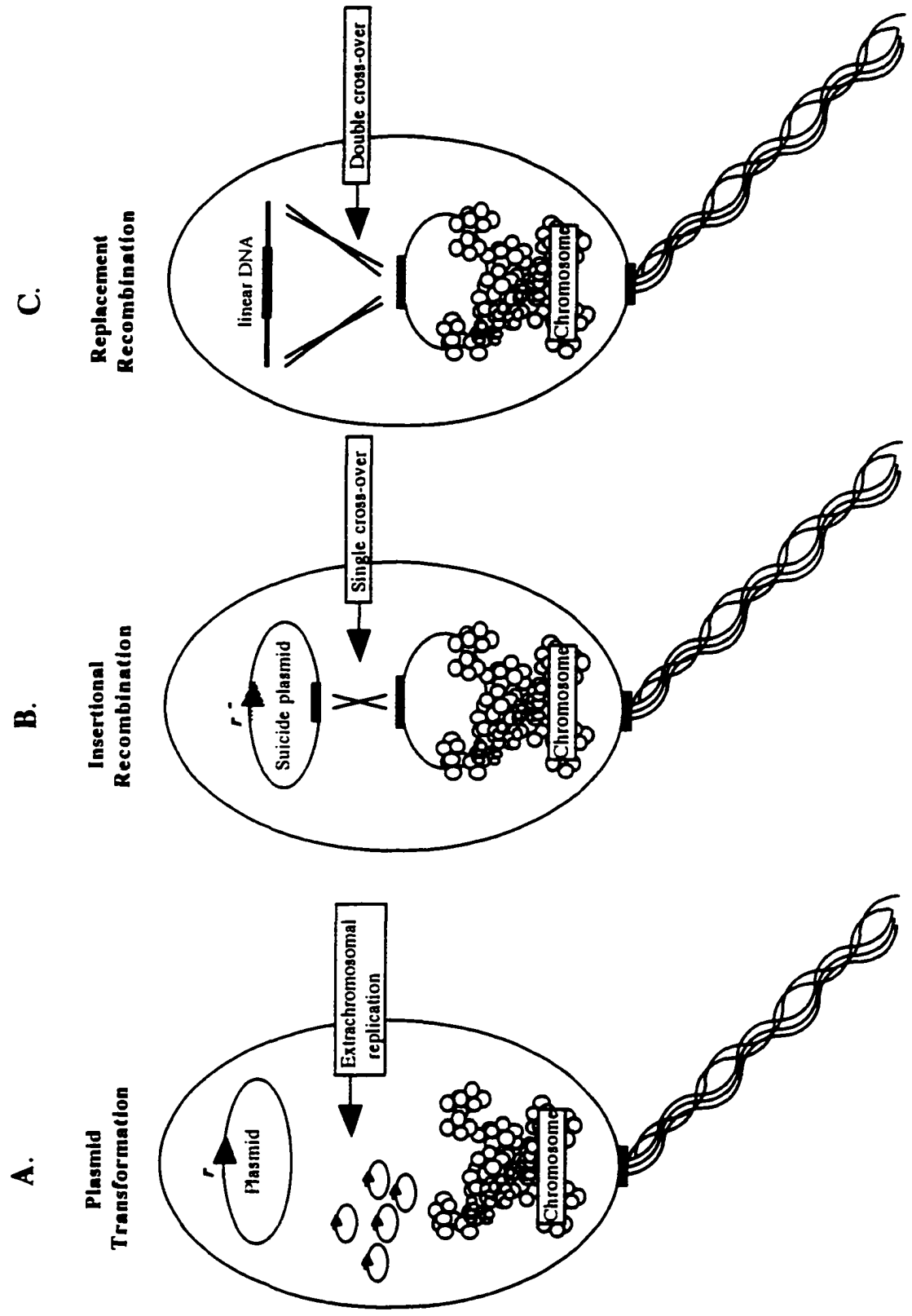
The ability to genetically manipulate an organism is required for an accurate molecular determination of virulence factors. *In vivo* genetic manipulation of a bacterium generally consists of two fundamental techniques: plasmid transformation and mutagenesis. These techniques utilize constructs consisting of supercoiled plasmids or linear fragments of DNA that possess genetic elements specific to the genetic manipulation desired, and are explained in detail below. The introduction of these DNA constructs into the bacterium can be accomplished by natural (conjugation and transduction) or artificial (electroporation or chemical) methods. In this study, we used electroporation to introduce a variety of plasmids (both replicative and non-replicative) and linear DNA fragments, and were able to demonstrate both plasmid transformation and site-directed mutagenesis.

Plasmid transformation is the introduction of a supercoiled plasmid harboring an origin of replication that is recognized by the replicational machinery of the bacterium and is subsequently maintained as an extrachromasomal fragment of DNA. Random mutagenesis is a group of methods (chemical, UV-light, transposon) designed to generate nonspecific mutations that are subsequently isolated by phenotype or biochemical means. Site-specific mutagenesis is the introduction of mutations into specific genomic loci, for which there are two techniques, replacement recombination and insertional recombination. Chapter Three of this work describes the first characterized mutant of any *Bartonella* generated by site-specific mutagenesis. Also mentioned in this section is natural mutant isolation, which was used in Chapter Four of this work to isolate the first characterized natural antibiotic resistant mutant. Each of these techniques are explained in detail below.

Figure 2. Depictions of three strategies of genetic manipulation.

A. Plasmid transformation is the introduction of a plasmid or cosmid harboring an *origin of replication* (*r*) that is recognized by the replicational machinery of the cell. Origins used in *B. bacilliformis* are RK2 (pEST) and REP (pBBR1MCS).

B. Insertional recombination occurs when a suicide plasmid (also referred to as an O-type gene targeting construct) recombines a single time with its cognate target. In this case, the *origin of replication* (*r* -) is not recognized, and thus the plasmid is termed a *suicide* plasmid. The entire construct is integrated into the chromosome by homologous recombination, both inactivating the targeted gene and inserting an antibiotic resistance cassette for selection. This strategy is employed in specific aim 2 and was utilized to demonstrate site-specific inactivation of the flagellin gene (chapter 3). **C.** Replacement recombination utilizes a linear fragment of DNA (also referred to as a Ω-type *gene targeting construct*), inherently incapable of extrachromosomal replication. A double cross over event can replace either a few bases (specific aim 3) or disrupt the targeted gene (specific aim 2).



1. Plasmid Transformation

The term plasmid transformation illustrates the event in which a plasmid harboring a recognizable origin of replication is introduced into the organism resulting in the maintenance of extrachromosomal DNA and synchronous marker expression. Thus, replicative plasmids enable introduction and maintenance of genes into an organism as extrachromosomal elements.

Shuttle vectors are a very useful tool of genetic engineering and genetic manipulation and were used in this research for transcomplementation of the flagellin mutant (Chapter Three), and introduction of the flagellin-promoted green fluorescent protein (Chapter Five). Plasmids were also used to manipulate the flagellin gene in targeting constructs (Chapter Two) and to clone and express *gyrB* (Chapter Four). Maintenance of shuttle vectors usually requires constant presence of the antibiotic corresponding to the resistance gene on the plasmid. In the course of this study, we determined that the most consistent replicon for high transformation efficiencies in *B. bacilliformis* was the broad-host-range vector pBBR1MCS and its derivatives, which contain the REP origin of replication (100, 101). Further, we demonstrate that this vector is a suitable shuttle vector for genetic experiments involving *Bartonella* species and *E. coli.*

Reschke *et al.* were the first to demonstrate plasmid transformation in *Bartonella* (formerly *Rochalimaea*) *quintana* by electrotransformation of the cosmid pEST (149). Subsequently, Grasseschi and Minnick were the first to demonstrate plasmid transformation in *B. bacilliformis* and it was reported that *B. bacilliformis* recognized the RK2 origin of replication but did not recognize pMB1, ColE1, or F origins (61). In this study, the plasmid pEST was introduced into high passage *B. bacilliformis* by electroporation, and high transformation efficiencies were reported (61). After numerous attempts to repeat this experiment with my own hands were futile, the host strain used by Grasseschi and Minnick was suspect for the high transformation efficiencies. To test this

hypothesis, extant stocks of *B. bacilliformis* containing pEST were cultured and subsequently 'cured' of the plasmid pEST by repeated culture in the absence of antibiotics. When cultured without antibiotic selection, replication of extrachromosomal plasmids is no longer necessary for survival (in most cases), and can be purged from the bacterium. These antibiotic sensitive bacterial clones are thus said to be 'cured' of the plasmid. This concept was utilized to isolate the transformation competent strain termed 'pEST-cured *Bartonella*' ('PCB', or 'JB584') which was used as the host strain in all subsequent genetic manipulation experiments (Chapters Two, Three, and Five). By curing a pre-transformed strain of *B. bacilliformis*, we found that an uncharacterized natural mutation occurred in the host strain used by Grasseschi and Minnick (61) that allowed foreign DNA to survive and replicate. Thus, 'PCB', designated *B. bacilliformis* strain 'JB584' was utilized in the genetic manipulation experiments described in Chapters Three and Five. An illustration of plasmid transformation is provided in Figure 2-A.

2. Random Mutagenesis.

A second general technique for genetic manipulation is random mutagenesis. There are various methods for accomplishing this procedure including UV irradiation, chemical mutagenesis and transposon-mediated mutagenesis. UV irradiation and chemical mutagenesis have the inherent disadvantage of possibly altering the genetic background of mutants generated by this approach. These anomalous secondary mutations are of concern when assessing the pathogenesis of an organism, for the virulence potential of a specific gene and gene product are in question relative to a wild-type background.

Transposons are often used as a nonspecific technique for mutagenesis provided there is a method for phenotypically or biochemically characterizing the mutants (50, 59). Transposon-mediated mutagenesis is the random insertion of a specific genetic element into a genome. The advantage of transposon-mediated mutagenesis over chemical and UV-light

induced mutations is that often a single gene can be mutagenized and characterized in a wild-type background. Disadvantages of transposon mutagenesis include random insertion, multiple insertion, and phenotype-directed isolation. This method was attempted for *B. bacilliformis* by electroporation mediated introduction of the plasmid pMGC20. This plasmid contains Tn*1545*- Δ3 and was not able to produce recombinants by this method (personal communication, M.F. Minnick, 1995). However, Dehio and Meyer recently reported successful conjugation between *E. coli* and *B. henselae* as a means of plasmid transformation as well as delivering Tn5 transposons on suicide plasmids for random gene inactivation (41). To date, conjugation has not been attempted by our laboratory.

3. Site-Specific Mutagenesis: Insertional Recombination.

A plasmid harboring an origin of replication that is *not recognized* by the recipient organism is termed a suicide plasmid, or suicide vector, for it will never replicate unless recombination with the genome occurs. If a *target* fragment of DNA is cloned into this suicide plasmid, it is termed an O-type gene targeting construct. Thus, expression of the resistance marker is a result of a single cross over, or single homologous recombination event with the genome. This single cross over effectively disrupts the target gene provided the target fragment in the suicide vector does not contain either end of the gene. Recombinants generated with these methods are referred to as 'knockouts' or 'null mutants' as expression of the target gene is interrupted. It is of course possible that illegitimate recombination can take place between the suicide vector and a portion of the genome other than the target. This strategy of genetic manipulation has been employed to inactivate a wide range of both prokaryotic and eukaryotic genes (68, 151, 159), and was used in Chapter Three to mutagenize the *B. bacilliformis* flagellin gene. An illustration of

insertional recombination is provided in Figure 2-B.

4. Site-Specific Mutagenesis: Replacement Recombination.

Alternatively, linear or Ω-type gene targeting constructs can be produced using one of two methods. In the first method, a two-step PCR amplification procedure utilizing overlapping primer sets generates a linear, Ω-type construct designed to specifically replace codons resulting in amino acid alterations. The utility of this technique is for generating and studying the effect of single amino acid substitutions. We used this technique to attempt demonstration of homologous recombination with the *gyrB* gene. However, we never had success even with the transformation efficient strain JB584 mentioned above. This method is illustrated in Figure 2-C.

The second general method of producing a linear, Ω-type gene targeting construct begins with the construction of a template plasmid in which an antibiotic resistance cassette is flanked by contiguous target sequences. Subsequent restriction digestion or PCR of the {target-(antibiotic resistance cassette)-target} generates a linear Ω-type construct designed to inactivate the target gene while simultaneously inserting a resistance cassette for selection. Antibiotic resistance can only result from a double cross over event between the contiguous target sequences and the genome. This method was attempted unsuccessfully to demonstrate homologous recombination with the flagellin gene and is illustrated again by Figure 2-C.

5. Natural Mutations.

Although naturally occurring mutations are not a means of genetic manipulation, these mutations can often provide information for the design of targeting constructs. By

culturing *B. bacilliformis* in the presence of 0.1 μg/ml coumermycin A_1, an antibiotic that targets the GyrB protein, we isolated naturally resistant mutants that occurred at a frequency of ~6 x 10^{-9}. Sequence analysis of *gyrB* from 12 coumermycin A_1-resistant (cou^R) mutants of *B. bacilliformis* identified single nucleotide transitions at three separate loci in the ORF. We hypothesized that introduction of PCR products incorporating the point mutations corresponding to coumermycin A_1 resistance would give rise to cou^R recombinants. This information was used to design several linear Ω-type gene targeting constructs using methods described by Samuels *et al.* (163). Unfortunately, we were unable to demonstrate homologous recombination by this technique in either wild-type or 'PCB' genetic backgrounds. However, in this study we isolated and characterized the first natural mutants of any *Bartonella* species. These data are reported in chapter four of this dissertation.

B. Homologous Recombination

Homologous recombination is process that results in modified genetic linkage relationships between genes or parts of genes. It is essential for chromosome segregation (in eukaryotic cells), maintenance of genomic integrity, and the generation of diversity (102). Mutational analysis in *E. coli* has identified at least 25 genes involved in the process, several of which have redundant functions (102). The genetic and biochemical models of homologous recombination are intricate and extensive. A cohesive explanation of the biochemistry would require at least another chapter in this dissertation. I will instead refer the reader to an excellent and thorough review of the biochemistry of homologous recombination by Kowalczykowski *et al.* (102). Explanations concerning homologous recombination are mentioned within this work where required for clarity.

III. RESEARCH SIGNIFICANCE AND GOALS

A system for genetic manipulation greatly facilitates investigation of the poorly understood virulence characteristics of the *Bartonella*. The overall goal of this project was to develop a system of genetic manipulation for *Bartonella bacilliformis* and to use this system to satisfy molecular Koch's postulates for the flagellum, a putative virulence factor. First, we hypothesized that a system for genetic manipulation could be developed and approached this hypothesis by experimenting with two separate loci of *Bartonella bacilliformis*; the *gyrB* gene and the flagellin gene.

Specific Aim 1: Optimize conditions for homologous recombination. After several initial attempts at mutagenizing the flagellin gene via an O-type construct it became apparent that there were genetic obstacles hindering *in vivo* homologous recombination. This initiated an investigation into methods of alleviating restriction as well as biochemical and metabolic manipulations which have been shown to increase the likelihood of homologous recombination. Insertional inactivation and allelic replacement strategies (in Specific Aims 2 and 3, respectively) were used to optimize the conditions for this event.

Results: After numerous biochemical and metabolic manipulations, two were able to promote homologous recombination. First, a strain of *Bartonella bacilliformis* was isolated by curing a plasmid pre-transformed strain. This strain, termed JB584, demonstrated a 2000-fold increase in plasmid transformation efficiencies and was successfully used as the host strain for genetic site-directed manipulation studies. Second, the addition of low levels of methionine may have inhibited epigenetic recognition of introduced foreign DNA or resulted in the induction of the SOS response, both of which should hypothetically increase homologous recombination efficiencies. The genetic constitution of JB584 as well as the enhancing properties of methionine still remain to be determined. These methods of optimizing the conditions are covered in Chapter Two of this

work.

Specific Aim 2: Demonstrate allelic inactivation of the *B. bacilliformis* flagellin gene. The flagellin gene has been cloned and sequenced, and is extant in our laboratory. Initially, subclones of the gene were made to produce a source of target DNA for the generation of gene targeting constructs. From this, both Ω-type (linear) and O-type (circular) gene targeting constructs were generated. Each construct was separately introduced by electroporation. Recombinants were selected by antibiotic resistance and molecular characterization included PCR analysis, SDS-PAGE, Western blotting, and TEM. Finally, complementation (introduction of the wild-type gene) of the flagellin mutant was used to attempt restoration of the wild-type phenotype, and thus construct strains for testing molecular Koch's postulates (Specific Aim 4).

Results: After numerous attempts we developed a system for site-specific genetic manipulation and were able to construct a mutant that no longer expresses flagellin. Restoration of the mutation by transcomplementation restored both expression and secretion of the flagellin subunits, the motility phenotype, and the virulence to wild-type levels. This is the first report of site-directed mutagenesis and transcomplementation for the *Bartonella*, as well as the first demonstration of molecular Koch's Postulates for the genus. These data, as well as the phenotypic and virulence properties of the strains are reported in Chapter Three of this work.

Specific Aim 3: Demonstrate allelic replacement of the *B. bacilliformis gyrB* gene. Single point mutations in the *gyrB* gene can confer resistance to coumarin antibiotics, thus providing a selectable phenotype and locus for replacement experimentation. Initially, the wild-type *B. bacilliformis gyrB* gene was cloned and

sequenced. We then isolated natural *gyrB* resistance mutants by growth in the presence of coumermycin A_1. Sequence analysis of these mutants provided data that were subsequently used to produce Ω-type (linear) gene targeting constructs which were introduced by electroporation. Finally, selection of *gyrB* recombinants in the presence of coumermycin A_1 was attempted.

Results: After numerous attempts using two separate *gyrB* loci, we were unable to demonstrate recombination-generated mutants. This strategy of replacement recombination differs from that in Specific Aim 2 in that this strategy requires two separate homologous recombination events (as opposed to one), and utilizes a linear fragment of DNA (in contrast to circular, supercoiled DNA molecules). This unsuccessful venture coincides with numerous attempts using a linear strategy to disrupt the flagellin gene, which were also abortive. Low levels of homologous recombination and/or the presence of an exonuclease are just two of the possible mechanisms that inhibited allelic replacement linear constructs.

Although we were not able to demonstrate allelic replacement of the *gyrB* gene, this research lead to several interesting results. The *B. bacilliformis* gene encoding the B subunit of DNA gyrase, *gyrB*, was cloned, sequenced, and characterized. The cloned *B. bacilliformis gyrB* was expressed in an *E. coli* -S30 cell-free extract, and was able to functionally complement an *E. coli* cou^R-ts gyrB mutant. We isolated and characterized background mutants of *B. bacilliformis* resistant to coumermycin A_1. Sequence analysis of *gyrB* from 12 cou^R mutants of *B. bacilliformis* identified single nucleotide transitions at three separate loci in the ORF corresponding to predicted amino acid substitutions. This study describes the first isolation and characterization of background mutants conferring natural resistance to an antibiotic for any *Bartonella* species and is described in detail in Chapter Four.

Specific Aim 4: Determination of phenotype and virulence of generated flagellin mutants. Preliminary data suggest that the bacterium requires flagella to invade erythrocytes. Scherer *et al.* determined that rabbit anti-flagellin antibodies decreased human erythrocyte invasiveness by nearly 98%, and adhesion by approximately 50% (164). In addition to motility, the flagella of *B. bacilliformis* has been speculated to be involved in attachment to erythrocytes (164, 179), and the surface exposure will make it a good vaccine candidate. Thus, we developed a system of genetic manipulation and used the system to construct flagellin mutants and complemented mutants (Specific Aim 1). We then assessed the phenotypic and virulence properties of the mutant and transcomplement by using protocols adapted from previous experimentation (14, 164, 179).

Results: The non-motile mutant was unable to swim through a *Bartonella* motility medium, which we developed, and demonstrated a decreased ability to adhere to human erythrocytes (Chapter Three). The complemented *fla*- mutant exhibited a swimming phenotype that was indistinguishable from wild-type and recovered the ability to adhere to erythrocytes (Chapter Three). These data demonstrate that the flagellum of *B. bacilliformis* is indeed a virulence factor used by the bacterium to adhere to human erythrocytes. This is the first demonstration of molecular Koch's postulates (49) for any of the *Bartonella*, and is described in Chapter Three of this dissertation.

CHAPTER TWO

Optimization of the Conditions for Homologous Recombination

I. INTRODUCTION

This section of the dissertation was not anticipated but became necessary after several initial attempts at mutagenizing the flagellin gene were unsuccessful. The overall goal of this research required the demonstration of homologous recombination, and it was realized early on that a combination of techniques would be necessary to achieve that goal. This section discusses the standard protocols and variations of these protocols that were employed to promote recombination or deter nuclease digestion of foreign DNA.

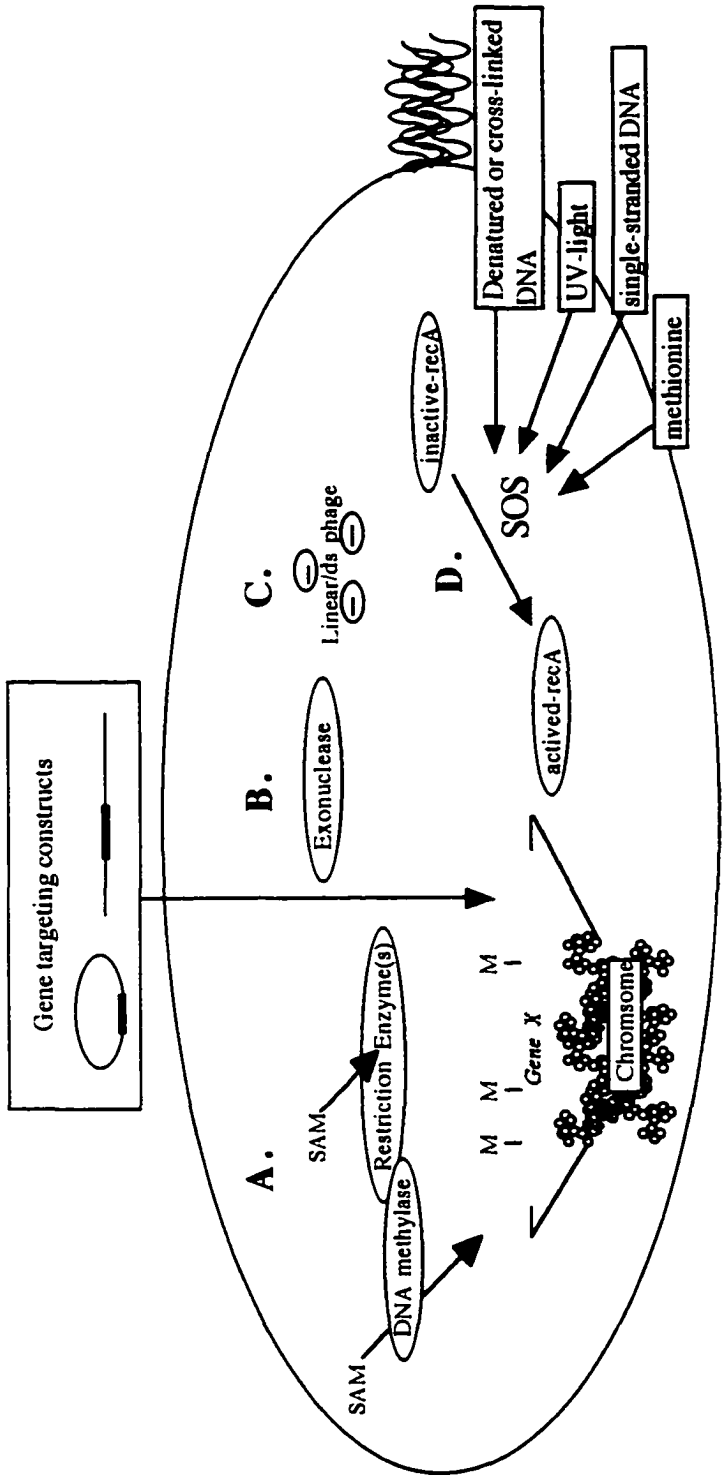
II. MATERIALS AND METHODS

A. Bacterial Strains and Culture Conditions.

B. bacilliformis was grown on heart infusion agar (Difco) supplemented with 5% defibrinated sheep erythrocytes and 2.5% filter sterile sheep serum (Quad Five, Ryegate MT) at 30°C in a water-saturated atmosphere. Where needed, antibiotic supplements for *B. bacilliformis* were 20 μg/ml kanamycin sulfate, 9 μg/ml chloramphenicol, and 0.1 μg/ml coumermycin A_1 (Sigma Chemical Co., St. Louis, Mo.) and coinciding with the experimental conditions were used individually or combined. Growth is usually observed after 3 days and cells were generally harvested after 5 days. All experimentation was performed in a laminar flow hood (Nuaire, Plymouth MN) to isolate this class II pathogen as well as to prevent bacterial and fungal contamination of cultures that will thrive on the rich medium.

Figure 1. An illustration of potential obstacles impeding homologous recombination.

A. The restriction modification system. The restriction enzyme recognizes foreign methylation patterns on DNA and subsequently fragments this DNA. In addition, S-adenoslymethionine (SAM) is the methyl donor for the cognate methylase and can be reduced by low level methionine exposure. **B.** Exonucleases could potentially be fragmenting the introduced DNA. **C.** The uncharacterized phage residing in *B. bacilliformis* might also affect the event. **D.** RecA, the mediator of homologous recombination, must be activated and available for the event to occur. Also depicted are the protocol modifications that initiate the SOS response and have been shown to indirectly result in the activation of RecA.



Escherichia coli strains used for propagation of cloned genes were grown overnight at 37°C in Luria-Bertani medium with standard antibiotic supplements where required (39).

B. Preparation and Manipulation of DNA

Chromosomal DNA from *B. bacilliformis* was prepared using CTAB (hexadecyltrimethyl ammonium bromide) by the methods of Ausubel *et al.* (9). Plasmid DNA extraction and isolation from *E. coli* for cloning was performed by the alkaline lysis procedure of Birnboim and Doly (18). Ultrapure plasmid DNA prepared for electroporation experimentation was prepared by Qiagen (Chatsworth, Calif.) Midi-prep kit or a 5 PRIME-3 PRIME (Boulder,CO) Perfect Prep kit as per the manufacturer's instructions. Restriction digestion of the DNA utilized numerous enzymes from several suppliers; Promega (Madison, WI), New England Biolabs (Beverly, MA), American Allied Biochemicals (Aurora, CO), and Gibco BRL (Gaithersburg, MD). The cloning of individual DNA fragments was accomplished by purifying DNA from ethidium-bromide-stained agarose gels by either a GeneClean kit (Bio 101, La Jolla, Calif.) or by a QIAquick kit (Qiagen). Ligation and transformation of the fragments into *E. coli* DH5 α was achieved by utilizing standard procedures (162). In addition to the standard procedures, T-vectors (Novagen, Madison, WI) were used for cloning PCR products, linkers (Promega), T4 polymerase (Promega), and S1 Nuclease (Promega) were used depending on the conditions of the cloning.

C. Electrocompetent Cell Preparation

1. Standard protocol

There are many factors involved in the preparation of electrocompetent cells that

influence the overall viability and efficiency of transformation (109). *B. bacilliformis* KC584 was grown as stated above and harvested with an ethanol-sterilized razor blade. Approximately 7 plates of 5-day-old cultured cells were harvested into 1 ml of 4°C heart infusion broth. The cells were subsequently washed 4 times with 1 ml ice cold 10% (v/v) glycerol-water with intermittent centrifugations at 2090 x g for 20 min at 4°C. The final bacterial concentration was measured with a Petroff-Hauser counter and adjusted to $1x10^{10}$ cells/ml with 10% glycerol.

2. Variations of standard cell preparation

a. Enhanced transformation rates have been correlated with high temperatures of growth (personal communication, K. Orzech, 1996). Abbott *et al.* have attributed this stimulation to a thermally-induced SOS response, thus activating the homologous recombination mediator, RecA (1). Therefore, as an anti-restriction enhancement, cells were grown at 37°C for 5 days prior to harvest for electrocompetency.

b. UV light-induced SOS induction has been correlated with temporary alleviation of host-controlled restriction in *Cyanobacterium*, *Bacillus*, and *E. coli* (47, 74, 75, 178, 187). As an anti-restriction enhancement, the cells were irradiated with UV light at approximately 32 J/m^2 with a standard 15-W germicidal bulb prior to harvest.

Unfortunately, the obvious disadvantage of UV-light induced SOS is the potential for nonspecific mutation of genes involved in virulence and thus render suspect the definitive genotype of mutants generated by this approach.

c. Growth in the presence of methionine has been shown to decrease the levels of S-adenosylmethionine (SAM) *in vivo* (78, 165). SAM is the methyl donor for DNA methylases and is also required for the function of Type I restriction enzymes (17). Thus, as an anti-restriction enhancement, the normal *B. bacilliformis* growth medium described above was supplemented with 5 mM methionine (Sigma).

D. Electroporation

1. Standard protocol

Electroporation of 44 μL bacterial suspension with 1-2 μl DNA were performed using a Bio-Rad Gene Pulser with 0.2-cm cuvettes (BioRad Laboratories, Hercules CA.) that have been chilled on ice for at least 1 h. The Gene Pulser configuration is as follows; exponential decay waveform set at a field strength of 12.5 kV/cm, a pulse time of 5 msec, and capacitance held constant at 25 μF. These parameters were optimized by Grasseschi *et al.* and are similar to those for other closely-related bacteria (61, 149).

2. Variations of the standard electroporation protocol

a. It has been demonstrated that RecA in *E. coli* is activated upon induction of SOS and that RecA is required for, and can even stimulate homologous, recombination *in vivo* (1, 107, 145). Furthermore, single-stranded DNA has been shown to induce the SOS response *in vivo* (56). Therefore, as an anti-restriction measure, electroporation mixtures described above were supplemented with nonspecific single-stranded oligonucleotides synthesized by Joan Strange at the Murdock Molecular Biology Facility at the University of Montana.

b. The standard 10% (v/v) glycerol-water employed in the electroporation suspension was modified several ways according to transformation procedures developed for other microorganisms (51). Addition of 1 mM $MgCl_2$ to the suspension and an increase in glycerol concentrations to 20% were utilized in an attempt to increase transformation efficiencies.

E. Post-Electroporation Treatment

1. Standard protocol

Immediately following electroporation, the cells were removed from the cuvette by resuspending in a sterile recovery broth modified from Benson *et al.* (heart infusion broth containing 0.5%(w/v) bovine serum albumin and 5% (v/v) sheep erythrocyte lysate)(4). The erythrocyte lysate was made by mixing one volume of defibrinated sheep erythrocytes with one volume of 4°C sterile dH_2O followed by centrifugation (4 min at 16 000 x g) to remove cellular debris. The cells and recovery broth were then transferred to a 15 ml sterile glass test tube and incubated for 14 h at 30°C in a water-saturated atmosphere, which corresponds to approximately two *B. bacilliformis* generation times (14). This incubation period was provided to allow time for recombination of the specific construct and for antibiotic resistance marker expression.

After this short incubation, cells were plated by transferring the 1 ml of suspension to a microcentrifuge tube, centrifugating at 2090 x g for 20 minutes to pellet the cells, and removing 900 µl of the supernatant. The cell pellet was resuspended in the remaining 100 µl and plated on heart infusion agar supplemented with 5% defibrinated sheep erythrocytes plus antibiotic, and subsequently grown at 30°C in a water-saturated atmosphere. Antibiotic supplements of 20 µg/ml kanamycin sulfate, 9 µg/ml chloramphenicol, and 0.1 µg/ml coumermycin A_1 were used individually or combined and coincided with the experimental conditions. According to Grasseschi *et al.*, colonies appear after 6 d of incubation in clones transformed with the replicative cosmid pEST (61).

2. Variations on the standard post-electroporation treatment

a. The 14 h of nonselective growth following electroporation has been shown to provide ample time for expression of resistance genes from a multicopy extrachromosomal locus. We hypothesized that the single copy nature of an integrated or chromosomal locus marker may require a longer period of nonselective growth to accumulate the resistance proteins, the nonselective growth time was therefore extended to 48 h.

b. As stated above in section C, low-level methionine exposure causes a reduction in the levels of S-adenosylmethionine (SAM) *in vivo* (78, 165). SAM is required for Type I restriction enzymes and is also the methyl donor for DNA methylases (17). A reduction in methylation should disrupt the constitutive restriction-modification system by inhibiting the methylation of chromosomal DNA and subsequently cause misinterpretation and restriction of self-DNA. This hypothetically results in an SOS response that, as stated above, enhances homologous recombination *in vivo* while concurrently activating RecA, the mediator of homologous recombination (145). For these reasons, the recovery broth was supplemented with 5mM methionine to stimulate a nonspecific, non-mutational (ie. UV-light) SOS induction.

F. Selection of the most efficient Gene Targeting Construct:

After several initial unsuccessful attempts at mutagenizing the flagellin gene using suicide plasmids, we were prompted to experiment with all known methods of *in vivo* homologous recombination. Generalized transposon mutagenesis and interplasmid recombination as well as the O-type and Ω-type targeting constructs were utilized in an attempt to demonstrate *in vivo* homologous recombination. Brief descriptions of these strategies are as follows:

1. Transposons are often used as a nonspecific technique for mutagenesis provided there is a method for phenotypically or biochemically characterizing the integrants (50, 59). We hypothesized that flagellin mutants could be isolated by loss of motility and therefore introduced pMGC20 that contains Tn*1545-Δ3* (168). As with UV-light-induced mutagenesis, this generalized system may cause anomalous mutations affecting virulence-related genes and render suspect the definitive genetic composition of mutants generated by this approach.

2. In an attempt to demonstrate interplasmid homologous recombination, the plasmid designated pPX was introduced into the pEST-containing strain generated by Grasseschi *et al.*(61). In brief, this plasmid has a pMB1 origin of replication that is not recognized by *B. bacilliformis*, and thus the chloramphenicol marker will only be expressed when interplasmid recombination occurs between pEST and the 6000bp *EcoRI/XmnI* pEST fragment cloned into pPX.

3. Three different O-type constructs (suicide plasmids), including pJMB1Δamp, pJPFLAG688 and pUB508 were generated with different kanamycin cassettes harboring individual regions of the flagellin gene. Each was subject to the alterations described in section G after standard procedures yielded no recombinants. An illustration of the three suicide plasmids is provided in Figure 2.

4. Several linear, Ω-type constructs were made. Template plasmids pFILA2 (kanamycin resistance) and pFLAGCAT (chloramphenicol resistance),were used to generate the Ω-type constructs via PCR amplification and used for flagellin gene recombination. This Ω-type flagellin inactivation requires the insertion of a large antibiotic resistance cassette via a double cross over. As with the O-type, these approaches were initially unsuccessful and were subject to the alterations described in section G, above.

Figure 2. Attributes of three of the O-type gene targeting constructs used in this study. Also termed suicide plasmids, these constructs were designed to inactivate the flagellin gene of *B. bacilliformis*. The hatched lines indicate flagellin fragments and the kanamycin cassettes are indicated as neomycin phosphotransferase (*npt*) type I or II. The noted origins of replication, pMB1 and R6K, are not recognized by *Bartonella* (Grasseschi et *al.*, 1994; Reschke *et al.*, 1990).

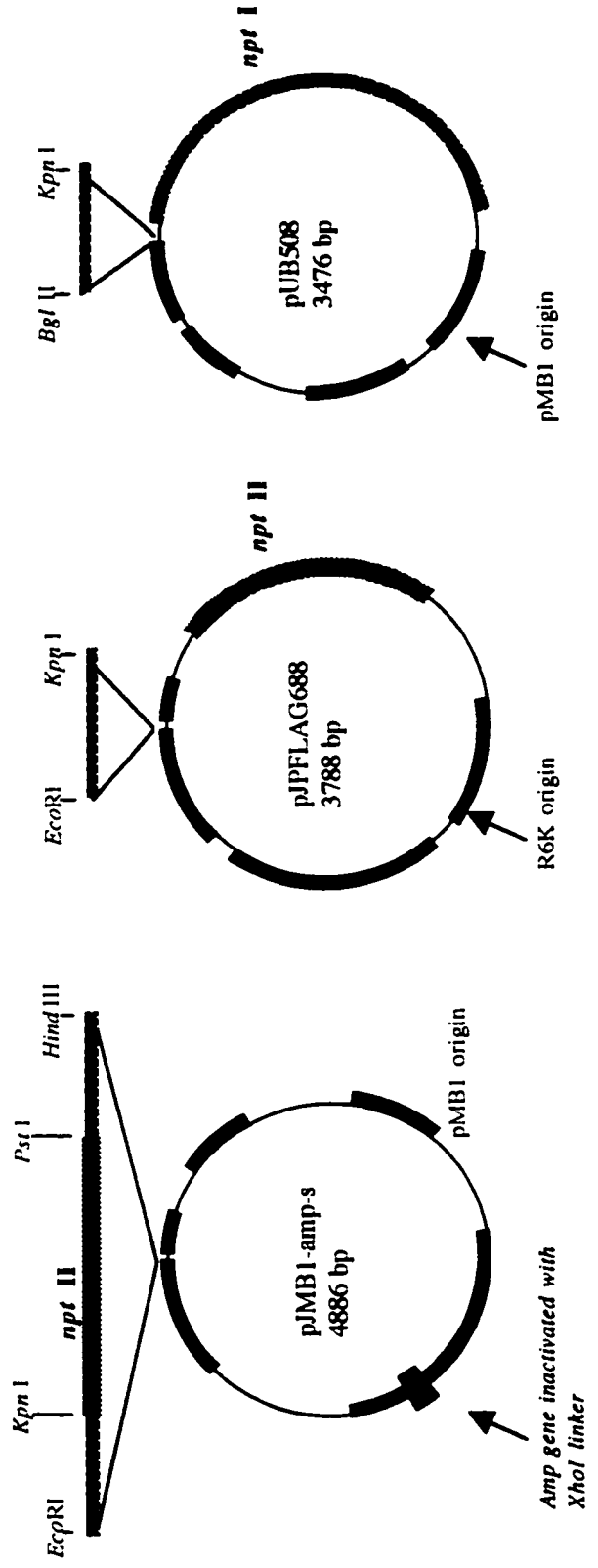


Figure 3. One of the strategies utilized to produce an Ω-Type gene targeting construct for replacement recombination with the flagellin gene. The template plasmid, pFILA2 and the amplimer set Fla5/Fla1200 were used to PCR amplify the linear construct illustrated. Hatched lines indicate flagellin sequence, and the kanamycin resistance cassette *nptI* is also shown. A double cross over event at a chromosomal locus will hypothetically insert the resistance cassette and interrupt the flagellin gene.

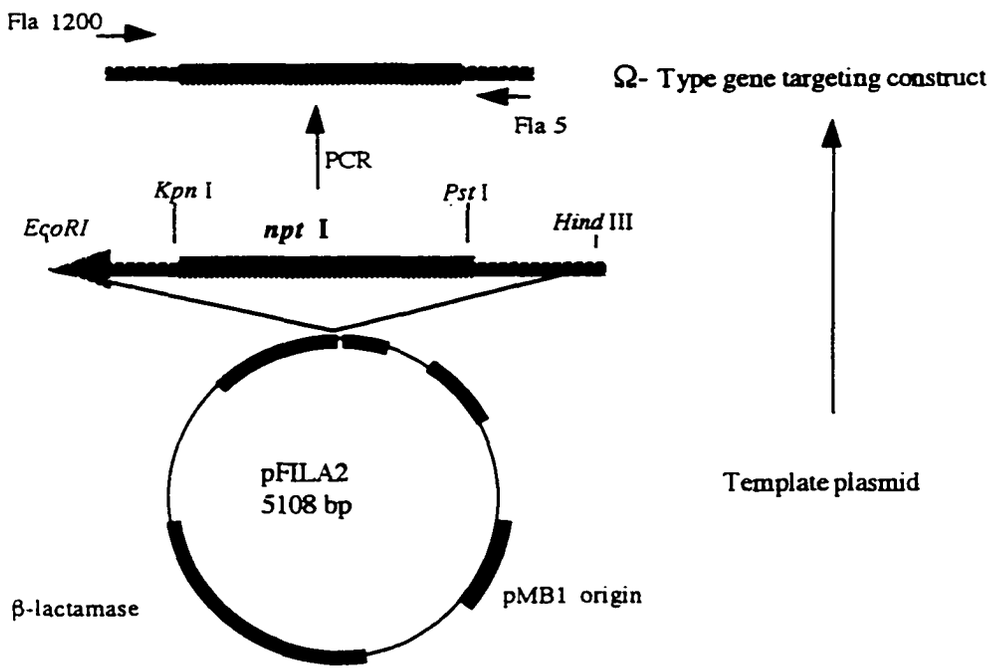


Figure 4. Generation of constructs Ω-Type gene targeting constructs for replacement recombination of the *gyrB* gene. The first step utilizes amplimers that overlap each other (672F / 642R) and harbor individual base substitutions. Upon polymerization, these amplimers become integrated into the fragments produced in A. A second PCR involves a 3 cycle thermocycler assembly of these fragments (in the absence of amplimers) and a 30 cycle amplification of the now assembled fragment with the amplimers 354F and 1128R. The result of the reactions is shown in B, a linear construct that has base changes (δ) designed to produce a single amino acid substitution shown to result in coumermycin A_1 resistance. (This strategy was adapted from Samuels *et al.*, 1994.)

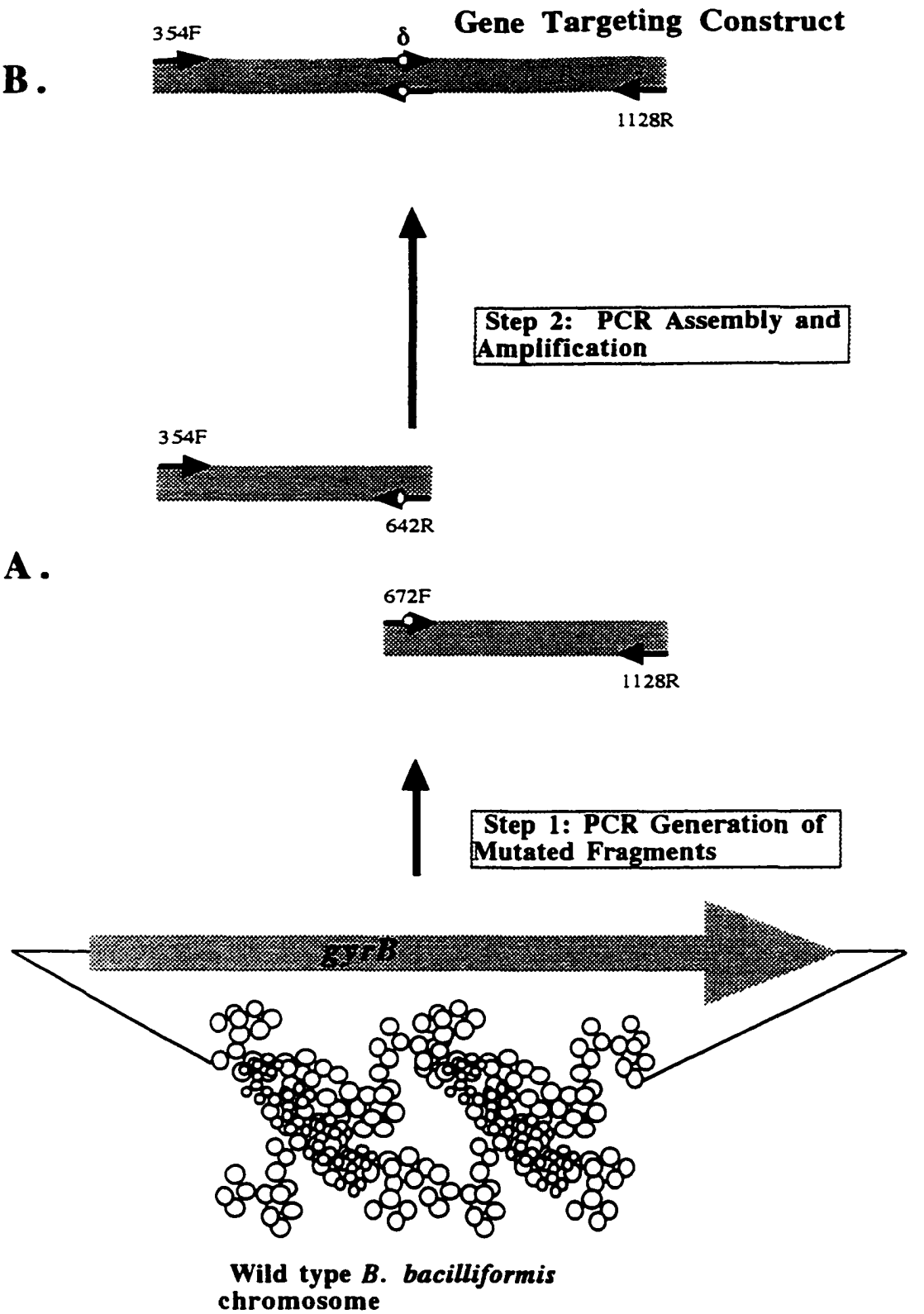


Figure 3 (p. 53-4) depicts one of the strategies used to generate linear constructs for replacement recombination with the flagellin locus.

The *gyrB* locus provides a means to demonstrate *in vivo* homologous recombination. By replacing a single codon (rather than inserting an antibiotic resistance cassette) in the *gyrB* gene via the Ω-type approach, an amino acid substitution results in coumermycin A_1 resistance. This Ω-type approach was used in replacement experimentation with the *gyrB* gene and is summarized in Figure 4 (p. 55-6).

G. Treatments to constructs

1. Standard protocol

After initial attempts to demonstrate *in vivo* homologous recombination were futile, modifications to standard protocols were utilized to promote the event. The following set of modifications focused on the treatment of the gene targeting construct prior to introduction by electroporation. Each treatment was designed to either promote homologous recombination via SOS induction of RecA or protect the construct from the endogenous restriction/modification systems.

2. Variations of standard construct treatment

a. Abbott *et al.* have demonstrated that insertional (O-type) homologous recombination is stimulated by UV-treatment of the suicide plasmid prior to introduction (1). Thus, as a protocol modification certain constructs were exposed to UV light at 300mJ/m^2 prior to introduction by electroporation.

b. In a similar notion, certain constructs were denatured by heating to 98°C in a microcentrifuge tube and immediately plunged into an ice bath. These denatured constructs were then introduced into the cells by electroporation. The hypothesis was that the single-stranded DNA would induce the SOS system and thus activate RecA.

c. Inherent in the Ω-type approach is the unavoidable introduction of non-methylated DNA via Taq polymerase. On the suggestion of P.F. Sparling (personal communication, 1996) several attempts were made to introduce a replicative vector with a fragment of the flagellin gene into *B. bacilliformis*, and allow the organism to modify the DNA *in vivo*. Subsequent preparation of the *B. bacilliformis*-modified construct was then restricted, purified (QiaSpin), and reintroduced into the organism by electroporation.

H. Isolation of a transformation competent strain

1. Standard protocol

The last application of a manipulation to the standard site-specific recombination protocols involved the strain of *B. bacilliformis*. Subsequent to the failed attempts mentioned, we examined the transformation rates of several strains of *B. bacilliformis*.

2. Variations of standard strains

a. In addition to the ATCC type strains 583 and 584, we obtained an apparent methylase minus strain of *B. bacilliformis* KC584 from L. Hendrix (Texas A&M University). The negative methylase character was demonstrated by the ability of normally non-recognizing restriction enzymes to fragment chromosomal preparations of this strain termed *KC584-long passage*. If the methylases are nonfunctional then it is very possible that the restriction system is also somehow altered, for a normally functioning restriction system would recognize the non-methylated DNA as foreign and the strain would never survive. Introduction of gene targeting constructs was performed on this strain.

b. Finally, the pEST-containing strain of *B. bacilliformis* generated by Grasseschi *et al.* was cured of the cosmid by growing on nonselective medium for 3 passages. Individual colonies were picked with a sterile needle and subcultured independently. Kanamycin sensitivity, Southern analyses (pEST and *nptI* probes), and PCR analyses (*nptI*

primer set) demonstrated that the pEST cosmid is no longer in this strain. It was thus nicknamed **PCB** for **p**EST **c**ured ***B**artonella bacilliformis* , and subsequently redesignated JB584.

III. RESULTS

By using the pUB508 suicide vector (Fig. 2), we were able to demonstrate site-specific homologous recombination and concurrent construction of the first characterized mutants of any *Bartonella.* The data concerning construction of the flagellin mutants, JB585 and JB586 are presented in Chapter Three of this work. Here, we were concerned with protocol alterations that would increase the likelihood of homologous recombination. Two independent alterations of the standard protocols may have promoted the event. First, as explained in section H, above, the PCB (JB584) strain of *B. bacilliformis* demonstrated a 2000-fold increase in plasmid transformation efficiencies with the replicative plasmid pBBR1MCS-2. Using this strain as the host strain for site-directed mutagenesis experimentation proved successful (Chapter Three). Second, the addition of methionine to the recovery broth may have also encouraged the homologous recombination event. However, the efficacy and underlying mechanism for this protocol alteration still remains to be determined.

III. DISCUSSION

The event of site-specific *in vivo* homologous recombination requires that the introduced DNA evade host exonucleases and endonucleases, find a genomic homolog, and recombine. The host is able to differentiate self from non-self DNA by an epigenetic pattern of methylation on the DNA. DNA that does not have the host-specific pattern is recognized by restriction endonucleases and subsequently digested, or 'restricted' from the cell. After numerous attempts using wild-type *B. bacilliformis* KC584 and KC583, not a

single recombinant was isolated.

To avoid this restriction, the obvious method was to methylate the introduced DNA such that it matched the host's pattern. We attempted to determine the methylation pattern by collaborating with a specialist in this field but this approach was too costly to pursue. We hypothesized that the restriction system was inhibiting survival of introduced foreign DNA, and therefore designed protocols that would hypothetically 'confuse' the self/non-self recognition. Low level methionine exposure should hypothetically reduce SAM and thus inhibit normal genomic DNA methylation. The cell would then begin to restrict its own genomic DNA. This induced misinterpretation between self and non-self may have allowed the foreign DNA to escape restriction and eventually recombine. The mechanism of methionine promotion of *in vivo* homologous recombination remains to be tested.

In contrast, the major factor that enhanced the *in vivo* homologous recombination event in *B. bacilliformis* was creation of the transformation efficient host strain PCB (JB584). The high efficiencies of plasmid transformation with pBBR1MCS-2 were independent of methionine. Again, a 2000-fold increase in plasmid transformation efficiencies prompted us to attempt mutagenesis experiments with JB584. It is obvious that the uncharacterized natural mutation(s) in this strain permits replication host-specific methylation, and subsequent extrachromosomal maintenance. Genomic DNA from JB584 retains the wild-type methylation pattern based on the inability of certain methyl-specific restriction enzymes to digest. This suggests that JB584 has a restriction system mutation that enables foreign DNA to survive. In contrast, it is interesting to note that the high-passage KC584 strain mentioned above (section H), which apparently lacks DNA methylation (based on the ability of certain methyl-specific restriction enzymes to digest) did not demonstrate an increase in plasmid transformation efficiencies. One would expect a methylase minus strain to accordingly be restriction minus to avoid restricting its own

DNA. The natural mutation(s) in strain PCB (JB584) remain to be determined.

CHAPTER THREE

Site-Directed Mutagenesis and Transcomplementation of the *Bartonella bacilliformis* Flagellin Gene.

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ABSTRACT

The lack of a system for site-specific genetic manipulation has severely hindered studies on the molecular biology of *Bartonella* species. We report the first demonstration of site-directed mutagenesis and transcomplementation for any *Bartonella* species, using the *B. bacilliformis* flagellin gene (*fla*) as a target. A highly competent strain of *B. bacilliformis* was isolated by curing a KC584 strain that had previously been transformed with the cosmid pEST. This strain, designated JB584, demonstrated a 2000-fold increase in transformation efficiencies, using the broad-host-range plasmid pBBR1MCS-2, relative to wild-type strains. A suicide plasmid, pUB1, was constructed with a pMB1 replicon and the neomycin phosphotransferase I gene (*nptI*). Recombinant DNA containing the cloned *fla* gene was isolated from a λ ZAP genomic library by screening with polyclonal anti-flagellin antiserum. An internal 508-bp fragment of the *fla* gene was then cloned into pUB1 to generate pUB508. The flagellin gene of JB584 was then insertionally mutagenized by electroporation-mediated introduction of pUB508 and subsequent homologous recombination. Characterization of two of eight kanamycin-resistant clones (JB585 and JB586) by PCR analysis and Southern blots indicated that allelic exchange with the suicide plasmid had occurred. Analysis by SDS-PAGE, immunoblots, and transmission electron micrographs showed that expression, secretion, and assembly of the 42-kDa flagellin polypeptide encoded by *fla* was also abolished. Finally, JB585 was

found to be nonmotile when compared to KC584 and JB584 using motility medium or phase contrast microscopy. In *trans* complementation was accomplished by transformation with a pBBR1MCS derivative, pBBRFLAG, containing the entire wild-type *fla* gene. Transcomplemented strains exhibited flagellin expression, flagellum assembly, and motility that was indistinguishable from wild type strains. These data conclusively show that pMB1 and pBBR1 replicons make suitable *B. bacilliformis* suicide and shuttle plasmids, respectively. Finally, in *in vitro* human erythrocyte adhesion assay, the *fla*- strain demonstrated a significant loss in adhesion relative to control strains, which strongly suggest that the flagellum is a virulence determinant involved in the pathogenesis of *B. bacilliformis*.

INTRODUCTION

The bartonellae are a unique group of intracellular bacteria that employ arthropod-mediated transmission and hemotrophy as common parasitic strategies. Five *Bartonella* species are presently considered agents of emerging infectious disease in humans (*B. bacilliformis, B. clarridgeiae, B. elizabethae, B. henselae,* and *B. quintana*). Diseases caused by *Bartonella* include endocarditis, cat-scratch disease, bacillary angiomatosis, bacillary peliosis and trench fever; several of which are manifested in AIDS patients (for recent reviews, see references 19 and 27). *B. bacilliformis* is the etiologic agent of Oroya fever and verruga peruana, and is indigenous to the Andes mountain range of South America. The primary phase of the disease, termed Oroya fever or Carrion's disease (13), is characterized by an acute fulminant anemia and has a 40% - 80% mortality rate in the absence of antibiotic treatment (17, 23). Humans present with this hematic phase of the disease within two to three weeks following inoculation of bartonellae into the bloodstream by the bite of a nocturnal sandfly (17). The chronic secondary phase of the disease, termed verruga peruana, develops weeks to months after inoculation and is

characterized by blood-filled cutaneous eruptions on the extremities (17). During this phase, *B. bacilliformis* invades vascular endothelial cells (11, 12, 25), a common event in other bartonelloses.

Although little is known of the molecular mechanisms involved in the pathogenesis of *B. bacilliformis*, reports have implicated the lophotrichous flagella (23) as a putative virulence factor in both adherence to, and invasion of, human erythrocytes. First, in the presence of anti-flagellin antiserum, erythrocyte association is attenuated by 50% and invasion is reduced nearly 100% relative to controls (40). Second, observation of *B. bacilliformis* interaction with erythrocytes by Namarski optics demonstrated that the bacteria use a boring or twisting motion that is accompanied by a remarkable deformation of the erythrocyte surface (2). Third, motile cultures of *B. bacilliformis* have been shown to have a higher rate of attachment and entry into erythrocytes (2, 26), and that the invasion requires energy exertion by the bartonella but not by the erythrocyte (44). In addition to flagellar-mediated mechanisms, two virulence factors termed deformin (26, 45) and IalA/IalB (29) have been reported to contribute to pathogenicity. These findings strongly suggest that a forced endocytosis entry into erythrocytes requires flagellar-mediated motility.

In order to provide irrefutable evidence regarding the virulence role of the flagella, or any other suspected virulence factors from a *Bartonella* species, a strategy for site-directed genetic manipulation is essential. In this report we describe a system of allelic exchange and transcomplementation for the *B. bacilliformis* flagellin gene and determine the effect of the mutation on motility and adhesion to human erythrocytes. This is the first report of site-specific mutagenesis and complementation for any *Bartonella*, and more importantly, the first demonstration of molecular Koch's postulates.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli* strains used for propagation of cloned genes were grown overnight at 37°C in Luria-Bertani medium with standard antibiotic supplements where required (6). Strains of *B. bacilliformis* and *E. coli* used or generated in this study are summarized in Table 1. *B. bacilliformis* was routinely grown on heart infusion agar (Difco, Detroit, MI) supplemented with 5% defibrinated sheep erythrocytes and 2.5% filter-sterile sheep serum (Quad Five, Ryegate, MT) at 30°C in a water-saturated atmosphere. Antibiotic supplements used in this study for *B. bacilliformis* were 25 μg/ml kanamycin sulfate, and 1 μg/ml chloramphenicol (Sigma Chemical Co., St. Louis, MO), which were used individually or combined depending upon experimental conditions. Colonies were usually observed 3 days after inoculation and cells were generally harvested after 5 days growth.

For testing the motility of *B. bacilliformis* strains, a *Bartonella* motility medium was devised. The defibrinated sheep erythrocyte supplement of normal growth medium was replaced with sheep erythrocyte lysate (2) at an equivalent 5% concentration to yield a translucent medium appropriate for this analysis. Second, the agar in the medium was reduced to 0.2% (w/v). Plates were poured and dried for 48 h at 22°C and for an additional hour at 45°C prior to inoculation to reduce surface moisture of the agar.

Preparation and manipulation of DNA. Chromosomal DNA from *B. bacilliformis* for use in DNA hybridization analysis or PCR analysis was prepared using CTAB (hexadecyltrimethyl ammonium bromide) by the methods of Ausubel *et al.* (1). Plasmid DNA extraction and isolation from *E. coli* for cloning was performed by the alkaline lysis procedure of Birnboim and Doly (3). Ultrapure plasmid DNA prepared for electroporation

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source/Reference
Strains		
B. bacilliformis		
KC584	Virulent wild-type strain	(4)
HG584	KC584 transformed with pEST [Km^r]	(16)
JB584	HG584 cured of pEST [Km^s]	This study
JB585	*fla* gene of JB584 interrupted by insertion of pUB508 [Km^r, *fla*$^-$]	This study
JB586	Isogenic to JB585 [Km^r, *fla*$^-$]	This study
JB686	JB585 flagellin gene complemented in *trans* by transformation with pBBRFLAG [Km^r, Cm^r, *fla*$^+$]	This study
E. coli		
DH5α	Host strain used for cloning and propagation of pUB508 and pBBRFLAG	(14)
Plasmids		
pUC18,19	Cloning vectors, Rep_{Ec}, Ap^r	(46)
pBK-CMV	Genomic library cosmid, Rep_{Ec}, Km^r	(42)
pBBR1MCS	*B. bacilliformis* shuttle vector, Rep_{Ec}, Rep_{Bb}, Cm^r	(22)
pBBR1MCS-2	*B. bacilliformis* shuttle vector, Rep_{Ec}, Rep_{Bb}, Km^r	(21)
pUCK18	pUC18 containing Km^r cassette(*nptI*), Rep_{Ec}, Ap^r, Km^r	(35)
pUCK19	pUC19 containing ~1500-bp *PstI* fragment of pUCK18, Rep_{Ec}, Ap^r, Km^r	This study
pUB1	Suicide plasmid. pUCK19 with a 1118-bp *BglII* fragment removed to delete *bla* gene, Rep_{Ec}, Km^r	This study
pUB508	Flagellin targeting suicide plasmid. pUB1 containing 508-bp *KpnI*/*BglII* fragment of *fla*, Rep_{Ec}, Km^r	This study
pAUL1	pBK-CMV containing 3800-bp *B. bacilliformis Sau*3AI fragment with *fla* gene, Rep_{Ec}, Km^r	This study
pFLAG3	pUC18 containing 1400-bp *Hin*dIII/*Eco*RI fragment of pAUL1 with *fla* gene, Rep_{Ec}, Ap^r	This study
pBBRFLAG	Complementation shuttle vector. pBBR1MCS containing 2158-bp *Hin*dIII fragment of pAUL1 with *fla* gene, Rep_{Ec}, Rep_{Bb}, Cm^r	This study

experimentation was prepared using a Midi-Prep kit (Qiagen, Chatsworth, CA) or a Perfect Prep kit (5 PRIME-3 PRIME, Boulder, CO) as per the manufacturer's instructions. The cloning of individual DNA fragments was accomplished by purifying DNA from ethidium bromide-stained agarose gels by either a GeneClean kit (Bio 101, Inc., La Jolla, CA) or by a QIAquick kit (Qiagen). Ligation and transformation of the fragments into *E. coli* DH5α was done by standard procedures (39). Plasmids used or constructed in this study are summarized in Table 1.

DNA Hybridization analysis. Total DNA from *B. bacilliformis* KC584, HG584, JB584 and pEST cosmid DNA were isolated, digested to completion with *Bam*HI, and then separated on an ethidium bromide-stained 1 % agarose (w/v) gel. The gel was then blotted onto nitrocellulose membrane (0.45-μm-pore-size; Schleicher & Schuell, Keene, NH) by the method of Southern (41) and baked for 1 h at 80°C. The cosmid pEST (35) was isolated with a Perfect Prep kit (5 PRIME-3 PRIME) and labeled by random primer extension (10) with the Klenow fragment of *E. coli* polymerase I (Gibco-BRL) and [α-^{32}P]dCTP (New England Nuclear, Boston, MA). The blot was probed overnight at 50°C with the ^{32}P-labeled pEST cosmid and washed as previously described (28). The blot was subsequently exposed for 1 h to X-ray film (X-Omat XAR-5; Eastman Kodak Co., Rochester, NY) to visualize hybridized DNA fragments.

Electroporation. *B. bacilliformis* strains were grown as stated above. Approximately seven plates of 5-day-cultured cells were grown as stated above and were harvested into 1 ml of 4°C heart infusion broth. The cells were subsequently washed four times with 1 ml ice cold 10% glycerol-water (v/v) with intermittent centrifugations at 2090 x *g* for 20 min at 4°C. The final bacterial concentration was measured with a Petroff-Hauser counter and adjusted to $1x10^{10}$ cells/ml with 10% glycerol. Electrotransformation was performed using a Bio-Rad Gene Pulser with 0.2-cm cuvettes (BioRad Laboratories, Hercules, CA) that had been chilled on ice for at least 15 min. In general, a 44 µl bacterial suspension ($1x10^{10}$ cells/ml) was combined with 1 to 2 µl DNA (1 to 44 µg/µl) and electroporated with an exponential decay waveform set at a field strength of 12.5 kV/cm, pulse time of 5 msec, and capacitance of 25µF as previously described for *Bartonella* (16, 35). Immediately following electroporation, cells were removed from the cuvette by resuspending in 1 ml ice-cold sterile recovery broth modified from Benson *et al.* (heart infusion broth containing 0.5% (w/v) bovine serum albumin 5% (v/v) sheep erythrocyte lysate and 5 mM L-methionine) (2). The cell-recovery broth suspension was then transferred to a 15 ml sterile glass test tube and incubated for 14 h at 30°C in a water-saturated atmosphere. This incubation period corresponds to approximately two *B. bacilliformis* generation times (2) and was provided to allow time for antibiotic resistance marker expression. Transformants were isolated by plating on standard *Bartonella* growth medium supplemented with kanamycin and/or chloramphenicol. Antibiotic resistant colonies usually appeared after 6 to 7 days of incubation at 30°C.

PCR and oligonucleotides. PCR amplification was achieved by using a GeneAmp 2400 Thermocycler (Perkin Elmer, Norwalk, CT) following procedures developed by Mullis *et al.* (32). Reaction mixtures contained 10mM Tris-HCl, pH 8.3, 50 mM KCl, 200 μM each deoxynucleotide triphosphate, 4 mM $MgCl_2$, 2.5 U AmpliTaq DNA polymerase (Roche Molecular Systems, Branchburg, NJ), 1 to 100 ng template DNA, and 0.1 μg of each primer. The reaction proceeded for 30 cycles of 1 min at 94°C, 1 min at 50-60°C (depending on calculated primer melting temperature), and 1 min at 72°C, with an initial 5 min denaturation at 94°C and a final 7 min extension at 72°C. Single-stranded oligonucleotide primers specific for the flagellin gene, Fla 5' (5'-AAGCTTTAGAGATTGTTTTGCAAA-3'), Fla 1300 (5'-AAATATTCTGGCTGCCCTGATTTGC-3') and the kanamycin cassette, NptI 5'(5'-AGCCACGTTGTGTCTCAAAATCTC-3'), NptI 3'(5'-CGTCCCGTCAAGTCAGCGTAATGC-3') were synthesized by The University of Montana Murdock Molecular Biology Facility. Target loci for each of the primers are schematically illustrated in Figure 1.

SDS-PAGE and immunoblotting. Whole-cell extracts of mutant strains and wild type controls were separated on sodium dodecyl sulfate-polyacrylamide gels (12.5% acrylamide) using procedures adapted from Laemmli (24). Protein bands were visualized by staining with Coomassie brilliant blue (39). For immunoblots, separated proteins were electrophoretically transferred from gels to nitrocellulose membranes (0.45-μm-pore-size; Schleicher and Schuell) by the methods of Towbin *et al.* (43). Immunoblots were developed using the procedures of Scherer *et al.* (40). Briefly, the blot was blocked with 0.3% Tween-20, 2% skim milk in phosphate buffered saline (PBS) pH 7.4 for 2 h at

22°C. Incubation with rabbit-anti *B. bacilliformis* flagella antisera was followed by a secondary horse radish peroxidase-conjugated goat anti-rabbit IgG (Sigma), and finally developed with PBS containing 4-chloro-1-napthol and H_2O_2.

Transmission electron microscopy. *B. bacilliformis* was grown as stated above and harvested into 1 ml heart infusion broth at 4°C. The cells were washed 3 times with 4°C 10% glycerol (v/v) with intermittent centrifugations at 2090 x g for 20 min at 4°C and resuspended in 10% glycerol. 15 µl aliquots of this suspension were placed on Formvar (Pelco, Tustin, CA) coated 300 mesh copper-palladium grids (Electron Microscopy Sciences, Fort Washington, Pa.) and incubated for 5 min at 22°C. The grids were then stained with 2% uranyl acetate (pH 7.0) for 3 min, de-stained with 1 M ammonium acetate (pH 7.0) for 3 min, and washed with deionized water for 1 min. The grids were then air-dried and observed at 75 kV with a model 7100 transmission electron microscope (Hitachi, Mountain View, CA) located at the University of Montana Electron Microscope Facility.

Erythrocyte adhesion assay. Seven plates of each strain (KC584, JB584, JB585, and JB686) were grown for 5 days on heart infusion agar supplemented with sheep erythrocytes, serum, and antibiotic (where required) as described above. *Bartonella* were subsequently harvested into 1 ml *Bartonella* liquid growth medium, or BLGM (minimum essential medium eagle, or MEM (Vitacell-ATCC, Rockville, MD), supplemented with 10% -SRBC lysate [as described above]), at 4°C. After gentle resuspension with a pipettor, each suspension was clarified of bacterial and agarose clumps by a 5 min incubation at 4°C after which the supernatant was transferred to a fresh 1.7 ml tube. Suspension volumes were equilibrated with BLGM to a total of 1.4 ml. Recently outdated human erythrocytes

(American Red Cross, Missoula, MT, Type B-positive) were washed five times in MEM at 4°C and diluted to ~1x10^9 cells/ml. *Bartonella* and erythrocytes were combined (in three replicates) to a final MOI of 1:1 in 24 well culture dishes (Falcon-Becton Dickinson, Lincoln Park , NJ, Type 3047) and incubated at 30°C for 7.5 hr. Reactions were then transferred to 1.7 ml tubes, washed twice with intermittent centrifugations of 20 sec at 4000 x *g* using 1 ml aliquots of MEM at 4°C. After the final wash, reactions were resuspended in 100 µl MEM, serially diluted, plated on heart infusion agar, and incubated at 30°C as described above. To determine the percent adherence, each of the stock *Bartonella* suspensions were also serially diluted and plated. CFU counts were made after 12 days growth.

Statistical analysis. The data reported for erythrocyte adherence are the means of three independent determinations ± standard errors of the means. Percentages of adherent bacteria are based on numbers of CFU/ml recovered from the adhesion reaction divided by CFU/ml values initially added to each reaction. Statistical significance of the data determined by pairwise comparisons using Student's T test where a value of $P < 0.05$ was considered significant.

RESULTS

Isolation of a highly competent strain. After numerous attempts to disrupt the flagellin gene in a wild-type *B. bacilliformis* strain (KC584) failed, we generated a highly competent strain by curing strain HG584. Grasseschi and Minnick (16) were the first to successfully transform *B. bacilliformis* by electroporation-mediated introduction of pEST, a cosmid containing an RK2 origin of replication and the neomycin phosphotransferase I

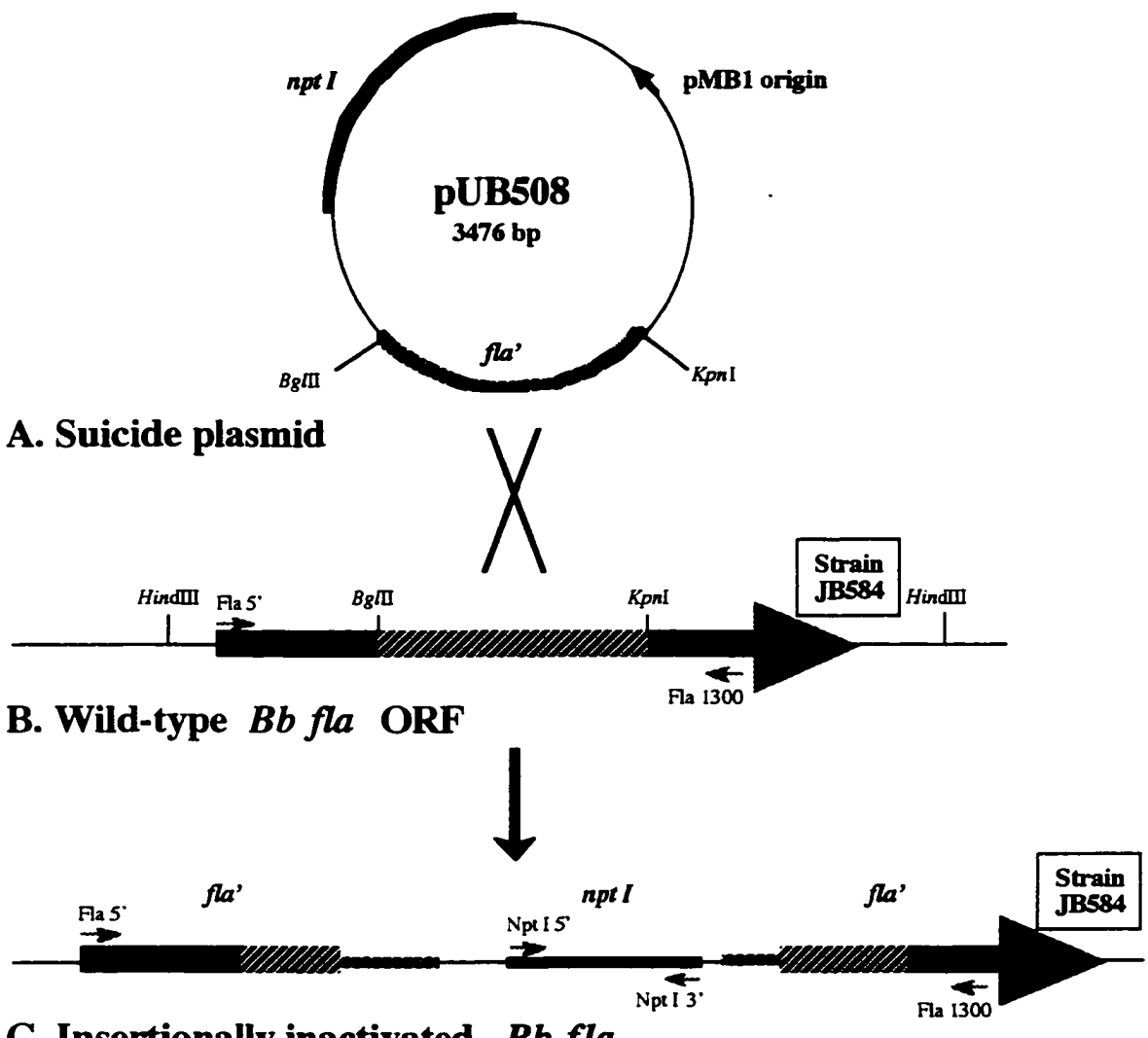
(*nptI*) gene encoding kanamycin resistance (35). This pEST-containing strain of *B. bacilliformis* (HG584) was passaged three times (15 days) in the absence of kanamycin sulfate. Six randomly selected clones were then subcultured independently and tested for sensitivity to kanamycin sulfate (25 μg/ml). All six clones exhibited wild-type sensitivities to kanamycin.

Two of these Kan^s clones were cultivated and chromosomal DNA was prepared by the methods of Ausubel *et al.* (1). DNA hybridization analyses with their chromosomal DNA using a ^{32}P-radiolabeled pEST probe was negative (data not shown), suggesting that the pEST cosmid was cured from these two strains. Further analysis of one of these strains (JB584) by PCR with *nptI* amplimers verified the absence of pEST (shown in Figure 2, lane 2). Thus, we were confident that JB584 was no longer kanamycin resistant, nor was pEST or *nptI* detectable.

The transformation efficiency of JB584 was subsequently assessed by electroporation-mediated introduction of pBBR1MCS-2. The plasmid pBBR1MCS-2, constructed by Kovach *et al.* (21), contains the REP origin of replication and the *nptI* kanamycin resistance cassette. Electrotransformation of pBBR1MCS-2 into JB584 demonstrated a 2000-fold increase in transformation efficiencies compared to KC584 and was used as the parent strain for further flagellin mutagenesis experimentation.

Cloning of *B. bacilliformis fla* and construction of suicide and complementation plasmids. The *B. bacilliformis* flagellin gene, termed *fla* was previously cloned, sequenced, and submitted to GenBank (Accession number L20677). The *fla* gene was simultaneously isolated by our laboratory from a λ ZAP expression library (Stratagene Cloning Systems, La Jolla, CA) with rabbit anti-flagellin antiserum. A

Figure 1. Schematic representation of site-directed *fla* disruption. The suicide plasmid pUB508 harbors a 508-bp *BglII*/*KpnI* internal fragment of the *B. bacilliformis* flagellin gene (*fla'*). The pMB1 origin of replication on pUB508 is not recognized by the replicational machinery of *B. bacilliformis* and thus expression of the *nptI* gene can only occur following recombination with the genome. The highly competent strain, JB584, contains the 1127-bp wild-type *fla* ORF. Note the position of the flagellin amplimers in relation to the gene. Electroporation-mediated introduction of pUB508 followed by homologous recombination results in site-directed insertion of pUB508 at the flagellin gene and generation of strain JB585 (*fla*$^-$, Kan^r). (Figure not drawn to scale.)



pBK-CMV cosmid clone containing the entire *fla* gene in a 3800-bp *Sau*3AI fragment was excised from the λ clone as per the manufacturer's instructions (Stratagene) and termed pAUL1. Restriction endonuclease sites from the GenBank sequence, restriction enzyme mapping, and nucleotide sequencing were used to map the *fla* gene within pAUL1.

pAUL1 was digested with *Hin*dIII, and the 2185-bp fragment containing *B. bacilliformis fla* was isolated by agarose gel electrophoresis (1% w/v) and purified by using a GeneClean II kit (BIO 101). Ligation of this fragment into the *Hin*dIII site of pUC18 (46) resulted in pFLAG3; a source of *fla* DNA fragments for constructing the suicide plasmid.

The flagellin targeting suicide plasmid pUB508 was constructed in several steps. Initially, a ~1500-bp *Pst*I fragment containing the *nptI* gene was subcloned from pUCK18 (35) into pUC19 resulting in pUCK19. Subsequently, the β-lactamase (*bla*) gene of pUCK19 was deleted by removing a 1118-bp *Bgl*II fragment and re-ligating to generate the suicide plasmid pUB1. Finally, a *Kpn*I/*Bgl*II fragment from pFLAG3 containing a 508-bp internal portion of the flagellin gene was cloned into pUB1 resulting in pUB508, the flagellin targeting suicide plasmid (Fig. 1). Verification of the pUB508 construct was accomplished by restriction endonuclease analysis and nucleotide sequencing (data not shown). Previous work had shown that the pMB1 ori of pUB508 was not recognized by the replicational machinery of *B. bacilliformis* (16, 35).

The complementation shuttle plasmid pBBRFLAG was constructed by cloning the 2158-bp *Hin*dIII fragment of pAUL1 into pBBR1MCS. The resulting plasmid, pBBRFLAG, contains the entire wild-type *fla* ORF and the REP *ori*. Ligation was confirmed by restriction digestion and PCR analyses (data not shown).

Characterization of *fla*- mutants by PCR. Eight kanamycin resistant clones were isolated following electrotransformation of pUB508 into JB584. The frequency of the homologous recombination event was approximately 1.8×10^{-8}. Genomic DNA from two of these clones was subjected to PCR analysis using three amplimer sets; *nptI*, *fla*, and insertion (refer to Figure 1 for a schematic representation of amplimer target loci). First, the *nptI* amplimer set (NptI 5'/NptI 3') generated the 983-bp *nptI* product in the mutant strains, JB585 and JB586 (Fig. 2, lanes 3 and 4 respectively), and was absent from the parent strain, JB584 (Fig. 2, lane 2). Thus, the kanamycin resistance in these strains was not a result of a background mutation. Second, the *fla* amplimer set (Fla 5'/Fla 1300) was used to determine whether the insertion of the suicide plasmid occurred at the *fla* locus. The parent strain, JB584 generated a 1304-bp product (Fig. 2, lane 5), whereas this product was absent from the JB585 and JB586 (Fig. 2, lanes 6 and 7, respectively). We assume that the elongation time for the amplification was not sufficient to generate the large 4.2-kb product expected from insertion of the suicide plasmid. Finally, the insertion amplimer set (NptI 5'/Fla 1300) was used to verify the chromosomal junction between *nptI* and *fla*. The ~2300-bp product generated from the mutant strains (Fig. 2, lanes 11 and 12) as well as its absence from JB584 (lane 8) and controls (lanes 9 and 10) confirms the successful site-directed flagellin disruption scheme illustrated in Figure 1.

Analysis of flagellin synthesis in mutant strains by SDS-PAGE and immunoblotting. The PCR analysis in Figure 2 confirmed that the wild-type 1127-bp *B. bacilliformis fla* ORF had been insertionally disrupted in strains JB585 and JB586. SDS-PAGE and immunoblotting were subsequently used to determine the extent of insertional inactivation on synthesis of the *fla* gene product in these mutant strains (Fig. 3). The wild-type 1127-bp *B. bacilliformis fla* ORF encodes a 42-kDa flagellin polypeptide (40).

Figure 2. Electrophoretic analysis of PCR products derived from *B. bacilliformis fla*- mutants.

PCR products were generated by amplification of chromosomal DNA obtained from parent and recombinant strains by using three amplimer sets. Amplimer sets designated *nptI* (NptI 5'/NptI 3'), *fla* (Fla 5'/Fla 1300), and insertion (NptI 5'/Fla 1300) were used to detect the kanamycin cassette, flagellin gene and insertional junction, respectively. The products generated from each of the amplifications are indicated on the left. The 983-bp *nptI* product is present in the mutant strains (lanes 3 and 4) and absent from the parent strain (lane 2). The 1304-bp *fla* product is present in the parent strain (lane 5) but is absent in the mutant strains (lanes 6 and 7). The ~2300-bp insertion product is present only in the mutant strains (lanes 11 and 12) and is absent from the parent strain (lane 8) and controls (lanes 9 and 10). Amplimer sets and the respective DNA templates used in this analysis are as follows: lane 1 (NptI 5'/NptI 3', Fla 5'/Fla 1300; [no template]), lane 2 (NptI 5'/NptI 3'; [JB584]), lane 3 (NptI 5'/NptI 3'; [JB585]), lane 4 (NptI 5'/NptI 3'; [JB586]), lane 5 (Fla 5'/Fla 1300; [JB584]), lane 6 (Fla 5'/Fla 1300; [JB585]), lane 7 (Fla 5'/Fla 1300; [JB586]), lane 8 (NptI 5'/Fla 1300; [JB584]), lane 9 (NptI 5'/Fla 1300; [pUB508]), lane 10 (NptI 5'/Fla 1300; [JB584 and pUB508]), lane 11 (NptI 5'/Fla 1300; [JB585]), lane 12 (NptI 5'/Fla 1300; [JB586]). PCR products were resolved by 1 % agarose gel electrophoresis and visulaized by ethidium bromide staining. DNA size standards are indicated to the right. Refer to Figure 1 for a schematic representation of amplimer target sites.

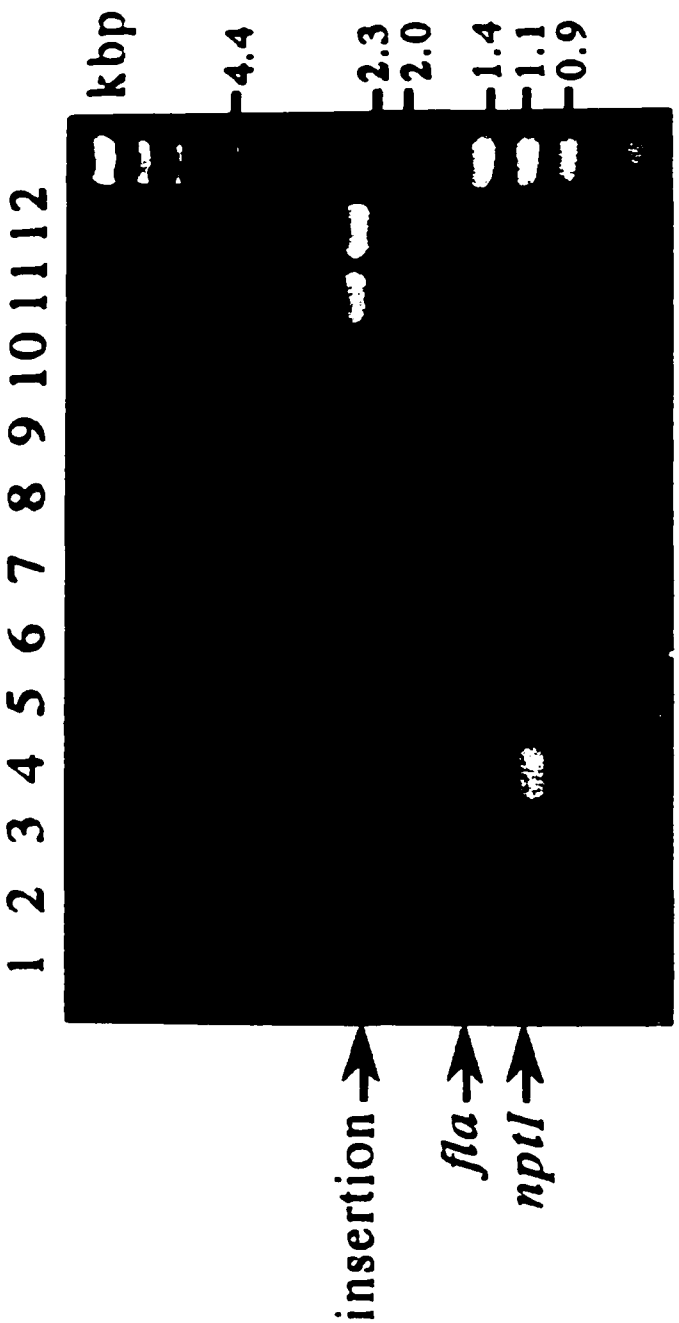
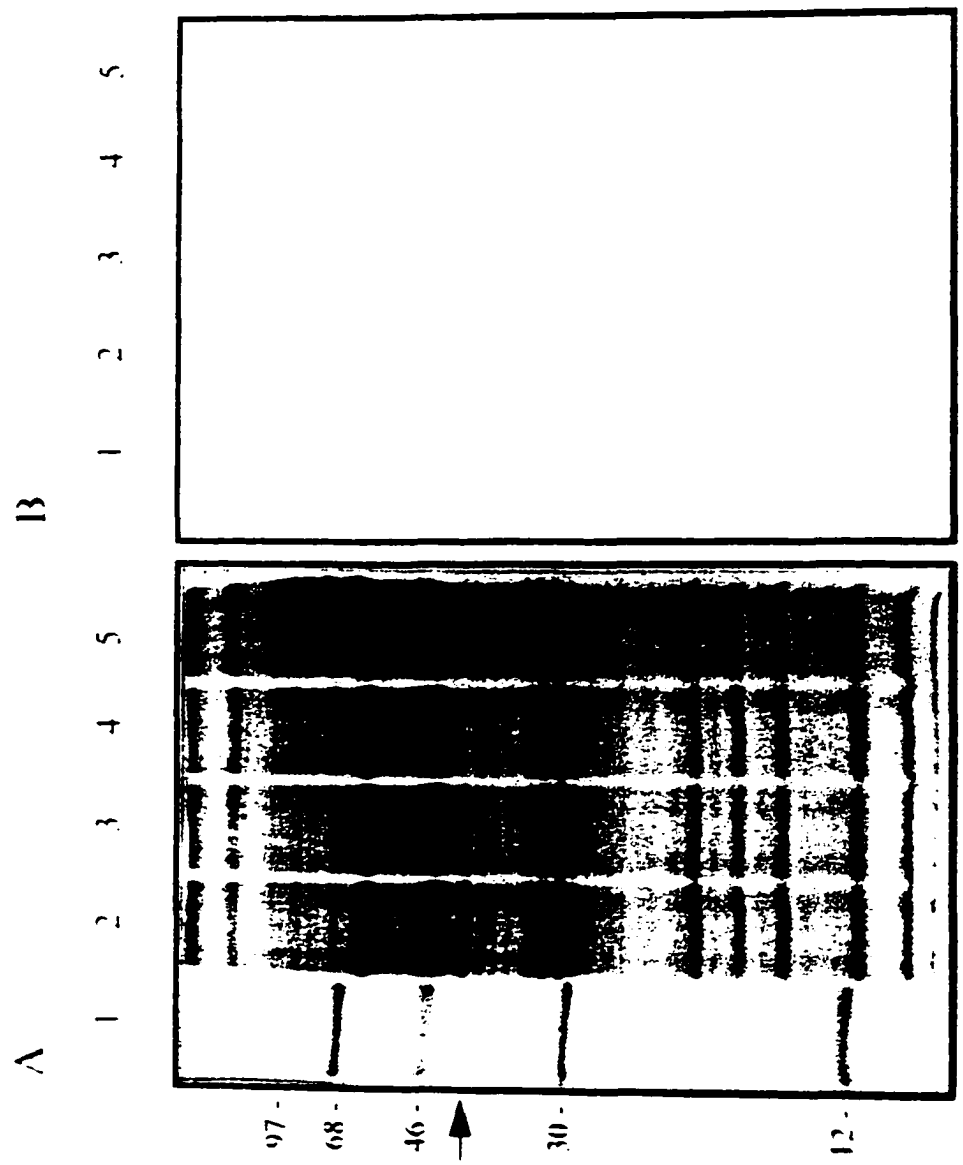


Figure 3. Flagellin synthesis in *B. bacilliformis fla-* mutants by SDS-PAGE and immunoblotting.

A. Whole cell extracts of parent and *fla-* mutant strains were separated on a 12.5% polyacrylamide-sodium dodecyl sulfate gel, and stained with Coomassie brilliant blue. The 42-kDa flagellin polypeptide, indicated by the arrow, is present in the parent strains KC584 (lane 2) and JB584 (lane 3), but is absent from the two kanamycin-resistant strains JB585 (lane 4) and JB586 (lane 5). B. Corresponding immunoblot incubated with polyclonal rabbit anti-flagellin antiserum indicating that expression of *fla* is completely abolished in the mutant strains. Lane 1 contains molecular mass standards (in kDa). The 42-kDa flagellin polypeptide is indicated by the arrow.



When whole-cell lysates were visualized on Coomassie brilliant blue-stained SDS-polyacrylamide gels, the flagellin polypeptide was clearly synthesized in strains KC584 (Fig. 3A, lane 2) and JB584 (lane 3). In contrast, the two *fla*- mutants JB585 (Fig. 3A, lane 4) and JB586 (lane 5) lacked the 42-kDa flagellin polypeptide, suggesting that *fla* expression had been disrupted. Immunoblot analysis was subsequently performed using rabbit polyclonal anti-flagellin antiserum as described previously (40). The immunoblot confirmed the presence of the flagellin polypeptide in KC584 (Fig. 3B, lane 2) and JB584 (lane 3), and also demonstrated that flagellin synthesis was abolished in both of the mutant strains JB585 (Fig. 3B, lane 4) and JB586 (lane 5). A truncated flagellin product was not observed in either strain.

PCR characterization of transcomplemented mutants. In an effort to eventually satisfy molecular Koch's postulates (9), we restored the wild-type flagellin phenotype to one of the mutants by in *trans* complementation. The plasmid pBBRFLAG contains the entire wild-type flagellin ORF, a chloramphenicol resistance cassette, and the REP origin recognized by the replicational machinery of *B. bacilliformis*. Electrotransformation of pBBRFLAG into JB585 followed by selection on medium containing 25 μg/ml kanamycin sulfate and 1 μg/ml chloramphenicol resulted in the isolation of strain JB686. Initial confirmation of the in *trans* complementation was accomplished by electrophoretic analysis of PCR products (Fig. 4). The *fla* amplimer set (Fla 5'/Fla 1300) was used to detect the presence of the *fla* gene, which was absent in the *fla*- mutant strain JB585 (Fig. 4, lane 2), but was present in both JB584 (lane 1) and the complemented mutant JB686 (lane 3). Finally, the ~2300-bp product generated by the previously described insertion amplimer set (NptI 5'/Fla 1300) demonstrates that the original chromosomal mutation was retained by the complemented mutant JB686 (Fig. 4, lane 4).

Analysis of flagellin synthesis in the complemented mutant by SDS-PAGE and immunoblotting. The PCR analysis in Fig. 4 confirmed that the wild-type *B. bacilliformis fla* ORF had been successfully reintroduced into the mutant strain JB585 resulting in the complemented mutant strain JB686. SDS-PAGE and immunoblotting were subsequently used to assay expression of the flagellin gene from the pBBRFLAG plasmid (Fig. 5). When whole-cell lysates of strain JB686 were visualized on Coomassie brilliant blue-stained SDS-polyacrylamide gels, the 42-kDa flagellin polypeptide was clearly evident (Fig. 5A, lane 3) indicating that *fla* gene expression was occurring from the plasmid locus. Immunoblot analysis was subsequently performed by reacting whole-cell lysates with rabbit polyclonal antiserum monospecific to flagellin. The positive signal confirmed the transcomplementation of *fla* expression from the plasmid in strain JB686 (Fig. 5B, lane 3).

Phenotypic characterization of strains by transmission electron microscopy. Transmission electron microscopy was used to visualize the secretion and assembly of the flagella in each of the strains (Fig. 6). Micrographs showed that wild-type strain KC584 (Fig. 6A) and competent strain JB584 (Fig. 6B) maintained the normal synthesis, secretion and assembly of flagellin polypeptides resulting in the wild-type lophotrichous flagella. Second, as evidenced by the lack of flagellar filaments, the flagellin ORF of strain JB585 has been insertionally disrupted and the synthesis, secretion and assembly of the flagellin polypeptide was abolished (Fig. 6C). Finally, the complemented mutant, strain JB686, not only synthesizes flagellin polypeptides from the plasmid locus, but these polypeptides were secreted and assembled (Fig. 6D) as in the wild-type strain (Fig. 6A). Therefore, the flagella phenotypes of the strains generated were consistent with the genotypes.

Figure 4. Detection of in *trans* complementation by PCR.

The wild-type phenotype was restored to the *fla* mutant JB585 by transcomplementation with pBBRFLAG resulting in strain JB686. PCR products were generated by amplification of chromosomal DNA obtained from parent and recombinant strains by using the same amplimer sets. Lane 3 indicates that the 1304-bp flagellin product (*fla*) has been complemented in *trans* in strain JB686. Furthermore, lane 4 demonstrates that the chromosomal locus of JB686 has retained the original ~2300-bp insertion. Amplimer sets and the respective DNA templates used in this analysis are as follows: lane 1 (Fla 5'/Fla 1300 [JB584]), lane 2 (Fla 5'/Fla 1300 [JB585]), lane 3 (Fla 5'/Fla 1300 [JB686]), lane 4 (NptI 5'/Fla 1300 [JB686]). PCR products were resolved by 1 % agarose gel electrophoresis and visulaized by ethidium bromide staining. DNA size standards are indicated to the right.

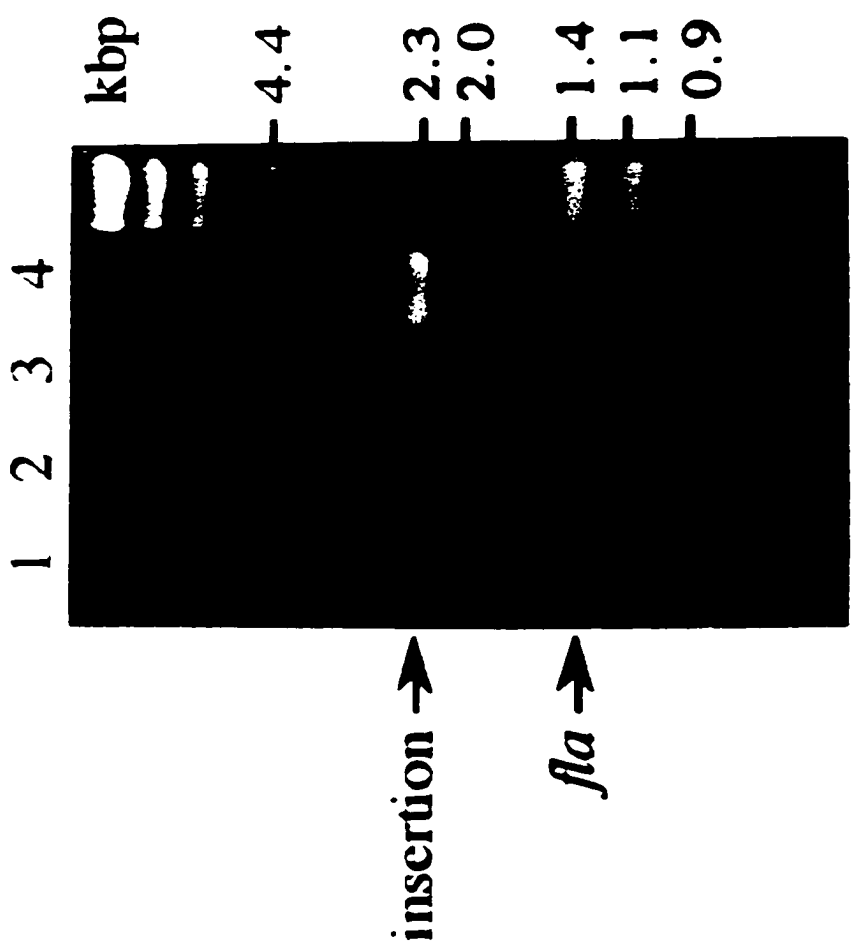


Figure 5. Flagellin synthesis in complemented mutants by SDS-PAGE and immunoblotting.

A. Whole cell extracts of parent, *fla*- mutant, and complemented strains were separated on a 12.5% polyacrylamide-sodium dodecyl sulfate gel and stained with Coomassie brilliant blue. The 42-kDa flagellin polypeptide is present in the parent strain JB584 (lane 1), and is absent from the kanamycin-resistant *fla*- mutant strain JB585 (lane 2). *fla* expression is detected in *trans* from the complementation plasmid pBBRFLAG in strain JB686 (lane 3). B. Cognate immunoblot reacted with polyclonal rabbit anti-flagellin antiserum indicating that flagellin synthesis is restored in the complemented strain. Molecular mass standards (in kDa) are indicated to the left and the 42-kDa flagellin polypeptide is marked by the arrow.

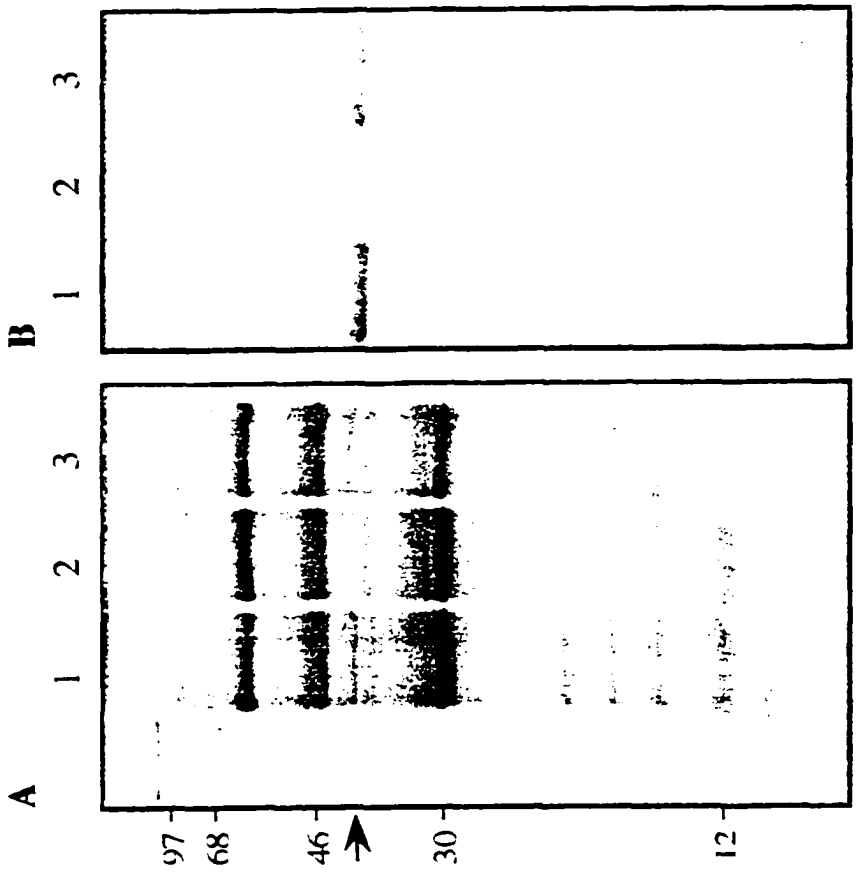
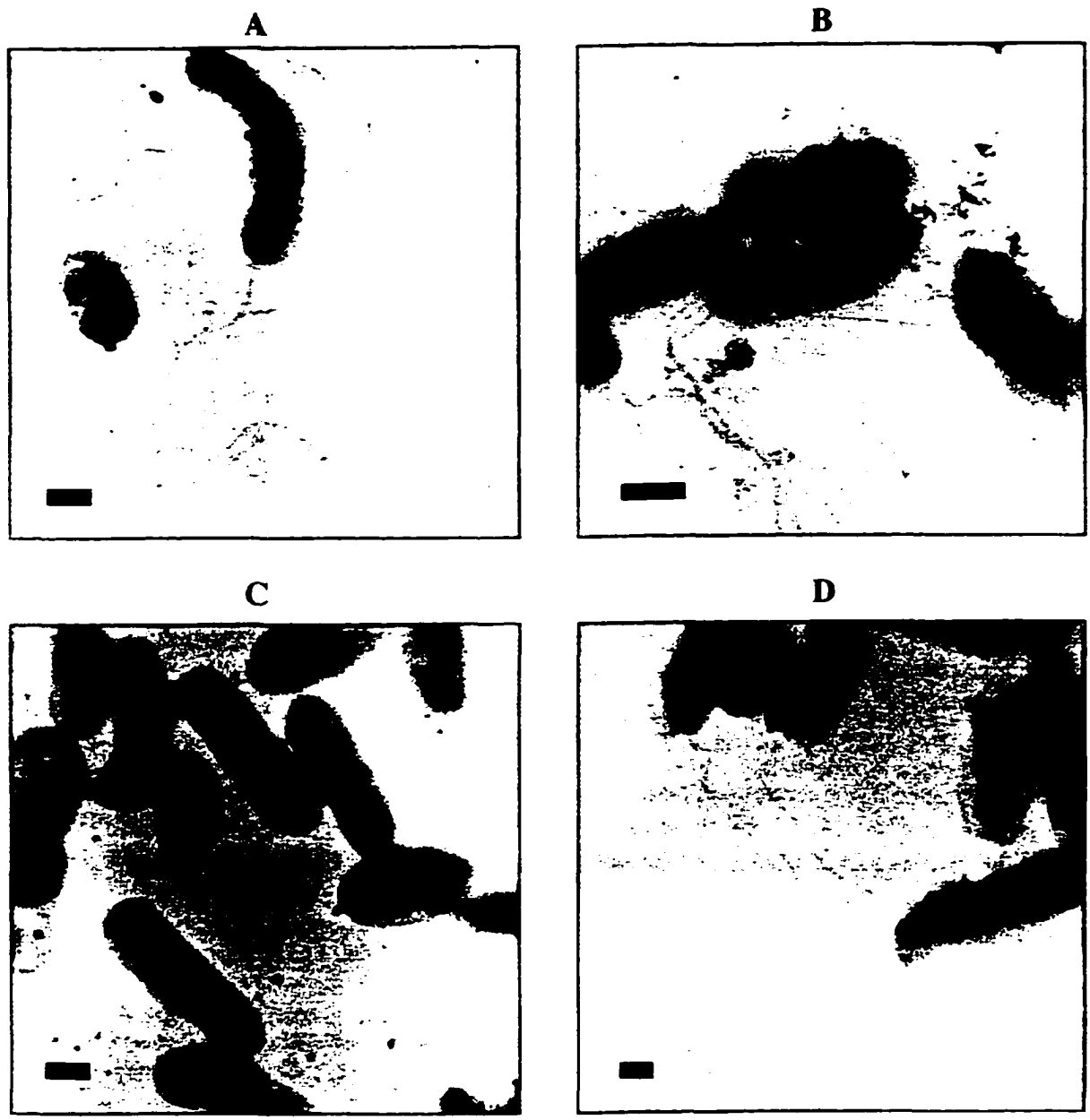


Figure 6. Phenotypic analysis of generated strains by transmission electron microscopy.

Transmission electron micrographs were prepared by staining 5-day-old cultures of *B. bacilliformis* with 2% uranyl acetate. Flagellin filaments are observed in panel A (wild-type strain [KC584]) and panel B (competent strain [JB584]) but are absent from the *fla*- mutant strain [JB585] in panel C. Expression, secretion and assembly of the flagella is restored in the transcomplemented strain [JB686] shown in panel D. (Bars = 0.5 μm)



Motility phenotypes of generated strains. Initially, phase contrast microscopy was used to examine wet-mounts of each of the strains. Using this method, a loss of motility was observed for strain JB585, whereas strains KC584, JB584 and JB686 appeared to have indistinguishable motility phenotypes. Motility was subsequently assessed by the ability of the bacterium to spread within a novel *Bartonella* motility medium. To develop the assay, we tested a wild-type strain in motility media with agar concentrations ranging from 0.2 - 0.8 % by stabbing the media with an inoculation needle from 5-day-old cultures. Incubation for 7 d at 30°C demonstrated that agarose concentrations of 0.6 and 0.8 % inhibited motility. At agarose concentrations of 0.2 and 0.4 %, however, motility was observed as a uniform halo of growth within the medium away from the site of inoculation. The four strains used in this study were subsequently tested in the same way using *Bartonella* motility medium containing 0.2 % agar. Multiple inoculations of each of the strains consistently generated the results shown in Figure 7. The wild-type strain KC584 (Fig. 7A) and competent strain JB584 (Fig. 7B) possess identical motility patterns indicating that the highly competent strain does not have an altered motility due to the transformation-curing process. The mutant strain, JB585 (Fig. 7C), was nonmotile, and did not produce a halo. The complemented mutant, strain JB686 (Fig. 7D), had a halo pattern that was indistinguishable from wild-type. Thus, the transcomplementation functionally restored wild-type motility to the mutant.

Erythrocyte adherence. Statistical analysis of the four strains tested demonstrate several significant results, as shown in Figure 8. First, the adhesion potential of the wild-type strain [KC584] was not significantly different from the transformation competent strain [JB584] (P=0.66). This suggests that the natural, uncharacterized mutation(s) of strain JB584 that enable high transformation rates do not affect the virulence. Second, there was a significant reduction in the adhesion ability of the *fla*- mutant [JB585] compared

to both KC584 (P =0.02) and JB584 (P =0.03). This strongly suggests that the flagellum plays a role in erythrocyte adhesion, and is a true virulence factor, either as a motive force to promote contacts via hypothesized adhesins such as fimbriae, or alternatively, the flagellum acts as an adhesin. Third, the complemented strain [JB686], expresses an intermediate phenotype, one in which there is an apparent increase in recovery compared to the *fla-* mutant, but does not fully restore adhesion potential when compared to the virulence phenotype of KC584 and JB584.

DISCUSSION

In vivo genetic manipulation of a bacterium generally consists of three fundamental techniques: plasmid transformation, random mutagenesis, and site-directed mutagenesis. The introduction of a replicative cosmid or plasmid, termed plasmid transformation, was first demonstrated in *Bartonella* by electrotransformation of the cosmid pEST into *B.* (at that time *Rochalimaea*) *quintana* (35). This was subsequently accomplished in *B. bacilliformis* by Grasseschi and Minnick (16), and it was reported that *B. bacilliformis* recognized the RK2 origin of replication but did not recognize pMB1, ColE1, or F origins. In the course of this study, we determined that the most consistent replicon for high transformation efficiencies in *B. bacilliformis* was the broad-host-range vector pBBR1MCS and it's derivatives, which contain the REP origin of replication (21, 22). Furthermore, we demonstrate that this vector is a suitable shuttle vector for genetic experiments involving *Bartonella* species and *E. coli.*

The second technique of genetic manipulation is random mutagenesis. Although there are various methods for accomplishing this procedure, Dehio and Meyer recently reported successful conjugation between *E. coli* and *B. henselae* as a means of plasmid transformation as well as delivering Tn5 transposons on suicide plasmids for random gene inactivation (7).

Figure 7. Motility of generated strains.

Plates of *Bartonella* motility medium (supplemented with antibiotics in mutant and complemented strains) were stabbed with 5-day-old cultures of each strain. The images shown are looking down on the progressing halo of motile bacteria moving outward from the central stab. Motility was indistinguishable in all of the strains except the flagellin mutant (C), which failed to form the characteristic halo pattern. Photos were taken 7 days after inoculation: panel A (wild-type strain [KC584]), panel B (competent strain [JB584]), panel C (*fla*- mutant [JB585]), panel D (in *trans* complement [JB686]).

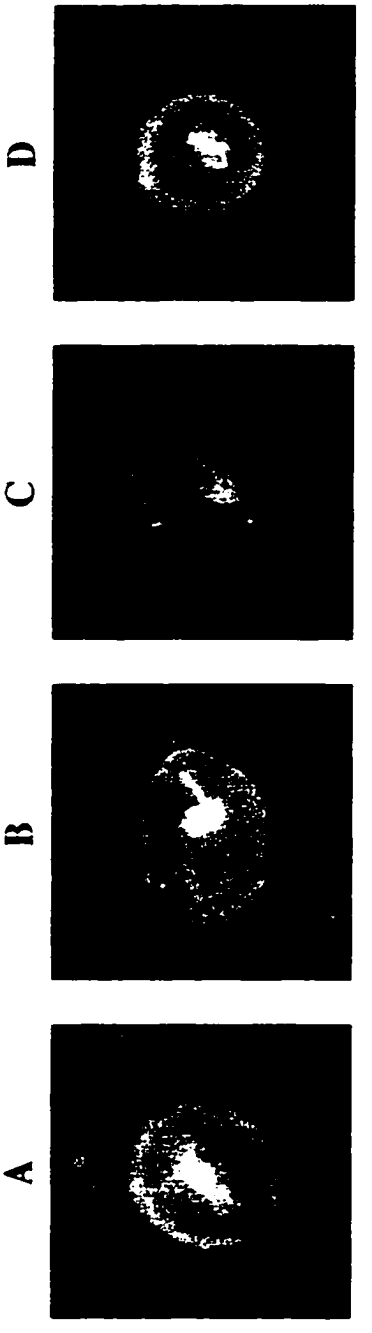
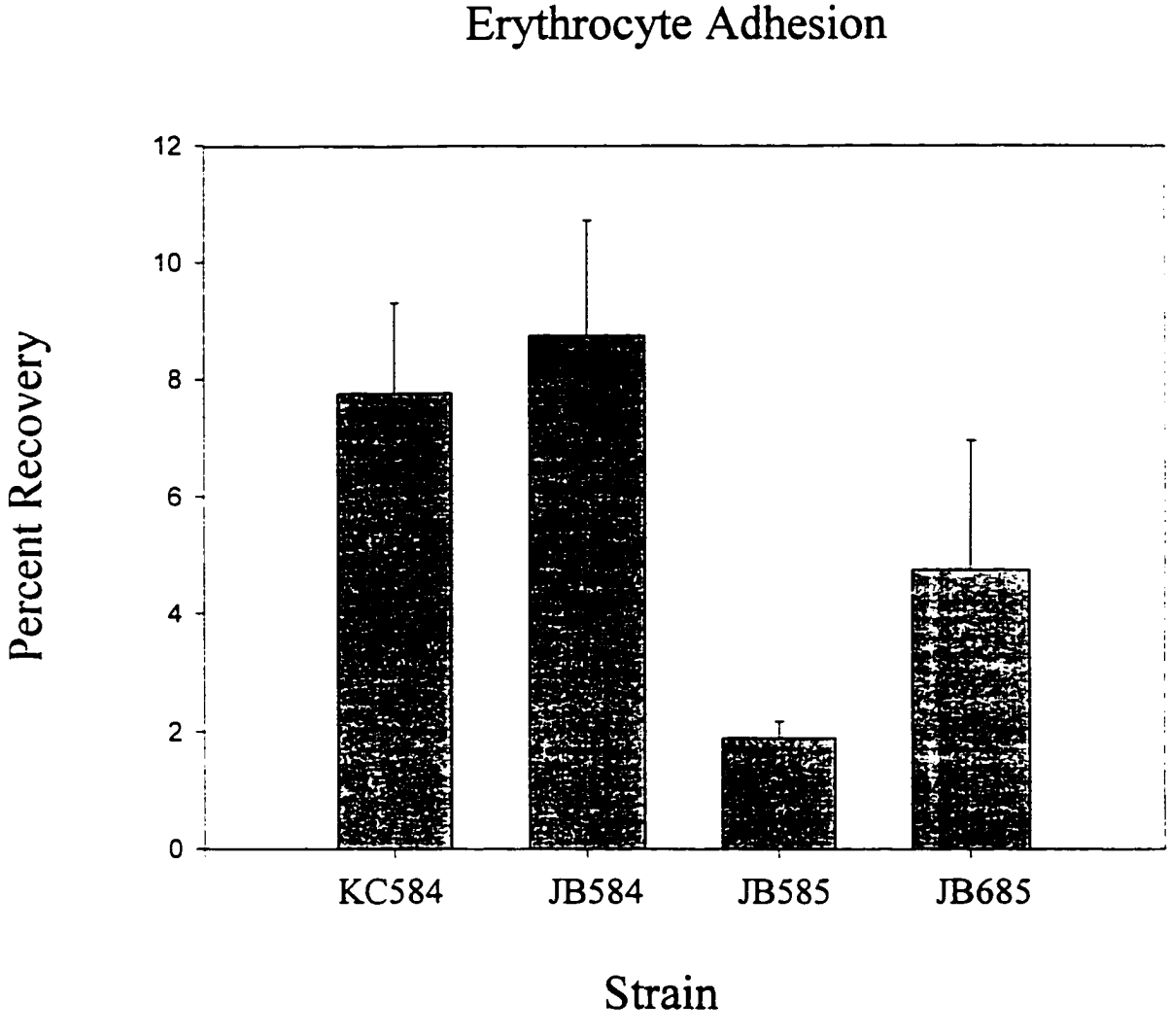


Figure 8. Human erythrocyte adhesion.

The data reported for erythrocyte adherence are the means of three independent determinations ± standard errors of the means. Percent recovery is based on numbers of CFU/ml recovered from the adhesion reaction divided by CFU/ml values added to each reaction. The adhesion potential of the wild-type strain [KC584] is not significantly different from the transformation competent strain [JB584] (P=0.66). Second, there was a significant reduction in the adhesion ability of the *fla*- mutant [JB585] compared to both KC584 (P =0.02) and JB584 (P =0.03). Third, the complemented strain [JB686], expresses an intermediate phenotype, not significantly different from strain KC584, JB584, or JB585.



The third method of genetic manipulation, the focus of this study, is site-directed mutagenesis, by which various methods can be used to alter or inactivate specific genes. The most commonly used method employs a suicide plasmid containing an internal portion of the target gene of interest and an origin of replication that is not recognized by the host cell DNA replication machinery. Expression of the resistance marker on the suicide plasmid can only occur after a homologous recombination event integrates the plasmid into the target site. Furthermore, transcomplementation of the mutation can be accomplished by reintroducing the wild-type ORF on a replicative plasmid or shuttle vector by plasmid transformation.

After several frustrating attempts at mutagenizing the flagellin gene using a suicide plasmid or linear DNA fragments failed, we realized that there were *in vivo* barriers impeding transformation in *B. bacilliformis*. This prompted us to try alternative methods for alleviating restriction/modification systems as well as biochemical/metabolic manipulations previously shown to increase the likelihood of homologous recombination. Although numerous manipulations were attempted, the generation of strain JB584 was a critical step towards the development of this system. The 2000-fold increase in transformation efficiencies exhibited by JB584 encouraged the use of this strain as the parent for successful mutagenesis experiments. However, the possibility exists that the mutation(s) enabling higher transformation efficiencies may also have an effect on virulence, although it did not effect adhesion. This possibility will be addressed in invasion and association assays currently underway.

The lophotrichous flagella of *B. bacilliformis* and the high degree of motility that it imparts has been implicated as a virulence determinant in several reports (2, 26, 40). In addition, *B. clarridgeiae*, an etiologic agent of cat-scratch disease, has also been reported as possessing a lophotrichous flagella (20). A dendrogram analysis of several flagellin sequences demonstrated that the *B. bacilliformis* Fla is most similar to *Azospirillum*

brasilense, *Rhizobium meliloti*, *Agrobacterium tumefaciens*, and *Caulobacter crescentus*, forming a cluster distinct from other flagellins (31). Where many bacteria possess multi-subunit flagella, the mutation data generated in this study suggest that the *B. bacilliformis* flagellar filament is composed of multiple polypeptide subunits encoded by a single flagellin gene. These data conclusively show that inactivation of the single *fla* gene completely abolishes the expression of the flagellum and generates a nonmotile and non-flagellated or 'bald' strain.

Flagellum-mediated motility is a common phenotype used by many pathogenic bacteria to colonize or gain access to host tissues or cells (34). Aflagellated or motility-impaired strains have decreased pathogenicity in numerous bacteria because of impaired colonization, adherence, or invasion, or by an uncharacterized method. Examples include *Borrelia burgdorferi* (38), *Campylobacter jejuni* (15), *Proteus mirabilis* (30), *Serpulina hyodysenteriae* (37), *Agrobacterium tumefaciens* (5), *Vibrio anguillarum* (33), *Vibrio cholerae* (36), *Salmonella typhi* (18), and *Helicobacter pylori* (8). Several reports have implicated uncharacterized motility-impaired *B. bacilliformis* as having a reduced pathogenicity (2, 26, 44). Scherer *et al.* were able to demonstrate that both erythrocyte invasion and association were decreased by treating the pathogen with anti-flagellin antiserum (40). Benson *et al.* reported the generation of natural nonmotile flagellated strains that could be maintained in either the motile or nonmotile form (2). However, we have not observed this phenomenon under the growth conditions described. Finally, naturally aflagellated *B. bacilliformis* have not been reported.

The ability to genetically manipulate an organism allows precise identification of the virulence determinants involved in pathogenicity. This identification must include a set of principles such as molecular Koch's postulates wherein alterations in pathogenicity are directly related to the presence or absence of the gene or gene product in question (9). These postulates were defined as follows: [1] The phenotype or virulence determinant

being studied should be associated with pathogenic strains of a species. [2] Specific inactivation of the gene(s) associated with the virulence trait should lead to a measurable loss in pathogenicity. [3] Replacement of the mutated gene should lead to restoration of the pathogenicity. Specific inactivation of the *B. bacilliformis* flagellin gene showed a significant loss in erythrocyte adherence compared to controls. Complementation of the mutation, however, only partially restores the adherence potential. Thus, this is a 'partial' demonstration of molecular Koch's postulates for the flagella. Inconsistent with this partial adherence phenotype is that the motility phenotype of the complemented strain [JB686] is indistinguishable from both the wild-type strain [KC584] and the transformation competent strain [JB584]. At first observation these data suggest that the flagellum is directly involved in the process of adhesion by acting as an adhesin itself, for motility seems independent of the extrachromosomal, multicopy nature of *fla* in the complemented strain [JB686]. However, three alternative explanations exist. First, the data generated in this study demonstrate that mutagenesis of *fla* completely abolishes the flagellin motility phenotype. Perhaps there is more than one component to the flagellum. Providing the *fla* ORF extrachromosomally, and at higher copy than wild-type could allosterically inhibit expression of a second component, which may be involved in erythrocyte adhesion. Second, the increased copy number or extrachromosomal status of the *fla* ORF may in some way alter expression, secretion, or assembly of the flagella such that motility (not apparent in our motility assay) or adhesins are affected. Third, the adherence assay was performed in the absence of antibiotic supplements, whereas the motility assay included the supplements. Perhaps the expression of the plasmid localized *fla* was diminished over the 7.5 hour adherence assay in a mechanism similar to the plasmid purge of a curing. It would be interesting to construct a *fla*+, motility- strain and include it in future studies.

As mentioned above, *Bartonella* have been shown to adhere to and invade both erythrocytes and endothelial cells, leaving several questions concerning the pathogenicity of

the flagella unanswered. This research has generated a system of genetic manipulation that will enable mutagenesis and complementation of other putative virulence determinants, as well as a method to determine the role of these factors in relationship to adherence.

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CHAPTER FOUR

Mutations in *Bartonella bacilliformis gyrB* confer resistance to coumermycin A_1.

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ABSTRACT

This study describes the first isolation and characterization of background mutants conferring natural resistance to an antibiotic for any *Bartonella* species. The *B. bacilliformis* gene encoding the B subunit of DNA gyrase, *gyrB*, was cloned and sequenced from overlapping phagemid clones derived from lambda genomic libraries by using radiolabeled probes to highly conserved regions of the gene. The *gyrB* ORF is 2079-bp and encodes a deduced amino acid sequence of 692 residues corresponding to a predicted protein of ~77.5 kDa. Sequence alignment indicates that *B. bacilliformis* GyrB is most similar to the GyrB protein from *Bacillus subtilis* (40.1 % amino acid sequence identity), and that it contains the longest N-terminal tail (52 residues) of any GyrB characterized to date; the only other tail of similar length (50 residues) occurs in *Mycobacterium tuberculosis* GyrB. The cloned *B. bacilliformis gyrB* was expressed in an *E. coli* -S30 cell-free extract, and was able to functionally complement an *E. coli* cou^R-ts gyrB mutant (strain N4177). We isolated and characterized background mutants of *B. bacilliformis* resistant to coumermycin A_1, an antibiotic that targets GyrB. Mutants were selected in the presence of 0.1 μg/ml coumermycin A_1, and occurred at a frequency of ~6 x 10^{-9}. Sequence analysis of *gyrB* from 12 cou^R mutants of *B. bacilliformis* identified

single nucleotide transitions at three separate loci in the ORF. The predicted amino acid substitutions resulting from these transitions are Gly124 → Ser, Arg184 → Gln, and Thr214 → Ala or Thr214 → Ile, which are analogous to mutated residues found in previously-characterized resistant *gyrB* genes from *Borrelia burgdorferi*, *Escherichia coli*, *Staphylococcus aureus*, and *Haloferax*. MIC analysis of the four types of cou^R mutants demonstrated that they are three to five times more resistant to coumermycin A_1 than the wild-type parental strain.

INTRODUCTION

Recent taxonomic re-classifications involving bacteria formerly constituting the *Rochalimaea* and *Grahamella* genera have rapidly expanded the number of species in the *Bartonella* genera (6, 9, 11, 30, 56). Of these 12 species, five are presently considered to be etiologic agents of emerging infectious disease in humans including *Bartonella bacilliformis*, *B. clarridgeiae* (30), *B. elizabethae*, *B. henselae*, and *B. quintana* (for recent reviews see 29 and 40). Hemotrophy and arthropod vector-mediated transmission are common parasitic strategies utilized by these small, gram negative, facultatively intracellular pathogens.

Bartonella bacilliformis is the etiologic agent of a unique biphasic disease that is endemic to South America, termed Oroya fever (1, 31). This geographic restriction coincides with the distribution of the sandfly vectors (*Phlebotomus* and *Lutzomyia* ssp.) (1, 18, 21, 31, 55). Humans are the only known reservoir, and in some areas of Peru over 60% of the asymptomatic population is seropositive for *B. bacilliformis* (28) and 5-10% are active carriers (31). Within four weeks following infection, the primary phase of the disease presents with an acute fulminating hemolytic anemia that has been reported to have as high as an 88% fatality rate in the absence of antibiotic therapy (20). During this

primary (hematic) phase nearly every erythrocyte is parasitized and upwards of 80% are lysed (26). Following resolution of the primary phase, the chronic secondary (tissue) phase, termed verruga peruana, presents with cutaneous lesions on the skin of the head and extremities which may occur for months to years (1, 18). Verruga peruana are clinically indistinguishable from the cutaneous lesions of bacillary angiomatosis (BA), caused by *B. quintana* and *B. henselae* (29).

Due to the lack of a system for site-specific genetic manipulation, few reports have been published concerning the molecular mechanisms involved in the pathogenesis, growth, and antibiotic resistance of *B. bacilliformis* (4, 16, 17, 31, 34, 36, 38, 41, 49, 54, 58). Therefore, we initially address this problem by molecularly characterizing the pathogen's *gyrB* gene. DNA gyrase is the bacterial type II topoisomerase responsible for introducing negative supercoiling into DNA (reviewed in 44 and 24) and it is the target of several types of antimicrobial agents. The holoenzyme is an A_2B_2 complex encoded by the *gyrA* and *gyrB* genes; the A subunit is responsible for DNA breakage and reunion, whereas the B subunit harbors the ATP binding site. The coumarin antibiotics coumermycin A_1, novobiocin, and chlorobiocin impede DNA replication by inhibiting the ATP binding and hydrolysis catalyzed by GyrB (35). Several reports have demonstrated that single point mutations in the *gyrB* gene confer resistance to coumarin antibiotics (12, 14, 23, 43, 46, 51) providing a locus and selectable phenotype for allelic exchange experiments.

In this study, we describe the isolation and characterization of the first background mutants of any *Bartonella* species, as well as the first characterization of an antibiotic resistance mutant. Analysis of coumermycin A_1-resistant mutants revealed single nucleotide lesions corresponding to specific amino acid substitutions in the N-terminal domain of GyrB. These mutations confer an approximately 5-fold increase in

coumermycin A_1-MIC relative to wild-type. In addition, we show that the *B. bacilliformis gyrB* can functionally complement an *E. coli gyrB* mutant. Finally, we discuss the position of the amino acid substitutions as they relate to recently solved high-resolution crystal structures and enzyme function (33, 57).

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli* strains used for propagation of cloned genes were grown overnight at 37°C in Luria-Bertani (LB) medium with standard antibiotic supplements when required (13). *B. bacilliformis* was routinely grown on heart infusion agar (Difco, Detroit, Mich.) supplemented with 5% defibrinated sheep erythrocytes and 2.5% filter-sterilized sheep serum (Quad Five, Ryegate, Mont.) at 30°C in a water-saturated atmosphere. Wild-type *B. bacilliformis* cultures were usually observed 3 days after inoculation and cells were harvested after 5 days of growth.

To isolate coumermycin A_1-resistant mutants, suspensions of 5-day-old cultures of *B. bacilliformis* KC583 were plated on heart infusion agar supplemented with 5% erythrocytes and 0.1 μg/ml coumermycin A_1 (Sigma Chemical Co., St. Louis, Mo.). Coumermycin A_1-resistant mutants were usually observed after 5 days growth and harvested after 7 days. Resistant colonies were picked with an inoculation loop and resuspended in 150 μl of heart infusion broth. Subsequent culture of the resistant mutants was achieved on heart infusion agar (with 5% erythrocytes) containing 0.04 μg/ml coumermycin A_1. Strains of *B. bacilliformis* and *E. coli* used or generated in this study are summarized in Table 1.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source/Reference
Strains		
B. bacilliformis		
KC583	Wild-type strain	[Brenner, 1991 #54]
CR1, 2, 6, 8, 9	KC583 $GyrB_{(Gly\ 124 \rightarrow Ser)}$ cou^R	This study
CR4, 7, 11, 12	KC583 $GyrB_{(Arg\ 184 \rightarrow Gln)}$ cou^R	This study
CR3	KC583 $GyrB_{(Thr\ 214 \rightarrow Ala)}$ cou^R	This study
CR5, 10	KC583 $GyrB_{(Thr\ 214 \rightarrow Ile)}$ cou^R	This study
E. coli		
HB101	Host strain used for cloning	Promega/[Boyer, 1969 #175]
TOP10F'	TOPO TA Cloning Kit host strain	[Invitrogen, 1997 #177]
N99	Complementation analysis *strA*, *galK*	[Menzel, 1983 #174]
N4177	Isogenic to N99 except *gyrB*221(cou^R), *gyrB*203(ts)	[Menzel, 1983 #174]
Plasmids		
pBK-CMV	Phagemid cloning vector	[Stratagene Cloning, 1993 #172]
pCR2.1-TOPO	Cloning vector	[Invitrogen, 1997 #177]
pGYRB1	pBK-CMV recombinant containing 5' portion of *B. bacilliformis gyrB* in a ~2000-bp *Sau*3AI fragment. Derived from λ-ZAP library.	This study
pGYRB2	pBK-CMV recombinant with a ~13-kb *Sac*I fragment containing the *B. bacilliformis gyrB*. Derived from λ-GEM 11 library.	This study
pGYRB3	pCR2.1-TOPO recombinant containing entire *gyrB* gene in a 2410-bp *Bam*HI fragment. Derived from TA cloning strategy.	This study

Preparation and manipulation of DNA. Chromosomal DNA from *B. bacilliformis* for use in DNA hybridization or PCR analyses was prepared using CTAB (hexadecyltrimethyl ammonium bromide) by the methods of Ausubel *et al.* (3). Plasmid DNA extraction and isolation from *E. coli* for cloning were performed by the alkaline lysis procedure of Birnboim and Doly (5), and plasmid preparations for sequencing were made with either a Midi-Prep kit (Qiagen, Chatsworth, Calif.) or a Perfect Prep kit (5 PRIME-3 PRIME, Boulder, Colo.) as per the manufacturer's instructions. Cloning of individual DNA fragments was accomplished by two distinct methods. First, both λ-ZAP Express (Stratagene Cloning Systems, La Jolla, Calif.) and λ-GEM 11 (Promega, Madison, Wisc.) genomic cloning systems were used as per the manufacturer's recommendations to obtain phagemid clones containing the *B. bacilliformis gyrB* gene for sequence analysis. Second, the TOPO TA Cloning Kit (Invitrogen, Carlsbad, Calif.) was used as per the manufacturer's instructions to obtain a plasmid clone containing the entire wild-type *gyrB* ORF for gene expression and functional complementation analyses. When required, DNA was purified from ethidium bromide-stained agarose gels or PCR reactions with either a GeneClean kit (Bio 101, Inc, La Jolla, Calif.) or by a QIAquick kit (Qiagen, Chatsworth, Calif.). Plasmids and recombinants used or constructed in this study are summarized in Table 1.

PCR and oligonucleotides. PCR amplifications were achieved by using a GeneAmp 2400 Thermocycler (Perkin Elmer, Norwalk, CT) following procedures developed by Mullis *et al.* (42). Reaction mixtures contained 10mM Tris-HCl, pH 8.3, 50 mM KCl, 200 μM each deoxynucleotide triphosphate, 4 mM $MgCl_2$, 2.5 U AmpliTaq DNA polymerase (Roche Molecular Systems, Branchburg, N.J.), 1-100 ng Template DNA, and 0.1 μg of

each primer. The reaction proceeded for 30 cycles of 1 min at 94°C, 1 min at 50-60°C (depending on calculated primer melting temperature), and 1 min at 72°C with an initial 5 min denaturation at 94°C and a final 7 min extension at 72°C. Single-stranded degenerate oligonucleotide primers (based on regions of conserved homology (25)) GYRB5 (5'-AARMGNCCNGGNATGTAYATHGG-3') and GYRB3 (5'-CCNACNCCRTGNARNCCNCC-3'), were synthesized by Gibco-BRL. Single-stranded oligonucleotide primers specific for the *B. bacilliformis gyrB* gene; GYRB-F (5'-CGCGGATCCCTGCGGAATAACAAATCATGGTG-3'), GYRB-R (5'-CGCGGATCCTATCGATAAAACGATCCATCTGGC-3'), LESION-F (5'-GCTGATTTGATTGATATAACATTGG-3'), and LESION-R (5'-TATAAATTTTTTCTGGGTCAAAAGC-3') were synthesized by the University of Montana Murdock Molecular Biology Facility.

DNA Hybridization analysis. Total DNA from *B. bacilliformis* strains KC583, KC584, and *E. coli* strain HB101 was isolated, digested to completion with *Bam*HI, and then separated on an ethidium bromide-stained 1% agarose (wt/vol) gel. The gel was then blotted onto a nitrocellulose membrane (0.45-μm-pore-size; Schleicher & Schuell, Keene, N.H.) by the method of Southern (50) and baked for 1 h at 80°C. The 2410-bp fragment used as the probe in this analysis was derived by PCR amplification using the amplimer set GYRB-F/GYRB-R and *B. bacilliformis* KC583 as template DNA. This 2410-bp PCR fragment was subsequently labeled by random primer extension (15) with the Klenow fragment of *E. coli* polymerase I (Gibco-BRL) and [α-^{32}P]dCTP (New England Nuclear, Boston, Mass.). The blot was probed overnight at 50°C with the ^{32}P-labeled 2410-bp PCR fragment and washed as previously described (39). The blot was subsequently exposed for 1 h to X-ray film (X-Omat XAR-5; Eastman Kodak Co., Rochester, N.Y.) to

visualize hybridized DNA fragments.

DNA hybridization was also used for probing two separate λ-genomic libraries to clone and sequence the *gyrB* gene. In these experiments, either the 300-bp PCR product derived from the degenerate amplimer set GYRB5/GYRB3 or the internal 1101-bp *Hin*dIII fragment were labeled by random primer extension. The λ-plaques were transferred to nitrocellulose filters and were probed overnight at 50°C with the corresponding ^{32}P-labeled fragment and washed as previously described (39). The blot was subsequently exposed for 1 h to X-ray film (X-Omat XAR-5) to visualize potential λ-recombinants.

***In vitro* transcription/translation.** Expression of *gyrB* was done using an *E. coli*-S30 cell-free *in vitro* transcription/translation (IVTT) system per the manufacturer's instructions (Promega). In brief, purified plasmid DNA was incubated with 10.0 μCi [^{35}S] methionine and the constituents of the IVTT kit for 2 h at 37°C. The reactions were stopped by placing the tubes in an ice bath for 5 min. An equal volume of Laemmli sample buffer (LSB)(32) was added and the sample was heated to 100°C for 10 min. After centrifuging for 1 min at 16,000 x g, a 15 μl aliquot was subjected to SDS-PAGE (12.5%, w/v acrylamide) analysis. The gel was vacuum-dried and subjected to autoradiography overnight (X-Omat XAR-5) to visualize labeled proteins.

In vivo complementation analysis. A plasmid containing the cloned *gyrB* (pGYRB3) and the respective cloning vector (pCR2.1-TOPO) were isolated and purified. These plasmids were separately introduced into strains N99 and N4177 by modifying the transformation procedure of Chung *et al.*(10) such that the culture temperature of N99 and N4177 was held below 30°C throughout the transformation procedure. Transformed

clones of N99 or N4177 containing either pGYRB3 or pCR2.1-TOPO plasmids were selected by incubation at 30°C for 16 h in the presence of 100 μg/ml ampicillin. Immediately thereafter, clones of each of the four transformants (N99[pCR2.1-TOPO], N99[pGYRB3], N4177[pCR2.1-TOPO], and N4177[pGYRB3]) were simultaneously replica-plated onto LB (supplemented with 100 μg/ml ampicillin), and incubated at either 30°C (permissive temperature) or 42°C (restrictive temperature) for 20 h. Both *E. coli* host strains (N99 and N4177) were replica-plated onto LB and LB 100 μg/ml ampicillin simultaneously for additional positive and negative controls, respectively. Replica-plated clones were scored after 20 h growth by estimating relative colony size.

Antibiotic susceptibility testing. MIC's were determined by two methods. First, determination of wild-type coumermycin A_1 MIC was achieved by plating 100 μl *Bartonella* suspensions containing 10^5 CFU/ml on heart infusion agar supplemented with 5% erythrocytes and coumermycin A_1 concentrations ranging from 0.01 to 1.0 μg/ml. Second, determination of MIC's for cou^R mutants was accomplished by an agar dilution technique previously described for *Bartonella* (34). Briefly, resistant strains were harvested after 5 days of incubation, washed and resuspended in phosphate buffered saline (pH 7.5). The suspensions were then equilibrated to a McFarland 0.5 standard at OD_{600}. Ten microliter aliquots were applied to heart infusion agar supplemented with 5% erythrocytes and coumermycin A_1 concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 μg/ml. The MIC is defined as the concentration of coumermycin A_1 at which no growth is detected following 7 d of routine incubation. MIC's were obtained by three independent determinations.

Nucleotide sequencing and computer analysis. The inserts of overlapping clones derived from λ-ZAP Express (pGYRB1) and λ-GEM 11 (pGYRB2) genomic libraries were sequenced separately to obtain the nucleotide sequence for the entire *gyrB* gene. Double-stranded phagemid or lambda phage DNA templates were prepared using a Plasmid Midi-Kit (Qiagen) or Lambda Kit (Qiagen), respectively, as per the manufacturer's instructions. These templates were then primed with M13 universal forward or reverse primers, or alternatively with synthetic oligonucleotides prepared with an Applied Biosystems (Foster City, Calif.) DNA synthesizer, Model 394. The nucleotide sequences for both DNA strands of the *gyrB* gene were then determined by the dideoxy chain-termination method of Sanger *et al.*(48) using a *Taq* DyeDeoxy Terminator Cycle Sequencing Kit as per the manufacturers instructions (Applied Biosystems). Sequencing was done on an Applied Biosystems Automated DNA Sequencer, Model 373A. Sequence data were compiled and analyzed using PC/GENE 6.8 software (Intelligenetics, Mountain View, Calif.) for restriction site determination and ORF identification; BLAST (2) for database searches; CLUSTAL-W 1.6 (53) for multiple sequence alignments; and BOXSHADE 3.2 (22) for sequence alignment formatting.

Nucleotide sequence accession number. The GenBank accession number for the *Bartonella bacilliformis gyrB* nucleotide sequence is U82225.

RESULTS

Cloning the *gyrB* gene. Two clones were required for sequence analysis of this gene. A positive plaque with the cloned *B. bacilliformis gyrB* gene was isolated from a λ-ZAP Express library (Stratagene) by probing with a [α-^{32}P]dCTP-labeled 300-bp PCR product

generated from *B. bacilliformis* KC583 template DNA using the degenerate oligonucleotide primers GYRB5 and GYRB3. A pBK-CMV phagemid clone was excised from the λ-ZAP Express clone and termed pGYRB1. Nucleotide sequence analysis revealed that only the 5' portion (1094-bp) of the *gyrB* gene was present in the ~2000-bp *Sau*3AI insert of pGYRB1.

To obtain the remainder of the sequence for *gyrB*, the 1101-bp *Hin*dIII fragment of pGYRB1 containing the 5' portion of the *gyrB* gene was labeled by random primer extension and used to probe a λ-GEM 11 genomic library (Promega) in hopes of obtaining a λ clone with a larger insert containing the entire *gyrB* gene. A second λ clone was identified and found to contain the entire *gyrB* gene in a ~13-kbp *Sac*I fragment by DNA hybridization. The *Sac*I fragment was excised and cloned into pBK-CMV to generate pGYRB2. The insert in pGYRB2 was used to complete the nucleotide sequencing of the wild-type *B. bacilliformis gyrB* gene.

The complete *gyrB* gene (2410-bp) was amplified from *B. bacilliformis* KC583 DNA using the amplimer set GYRB-F/GYRB-R and cloned into pCR2.1-TOPO. This *gyrB* recombinant was designated pGYRB3.

Nucleotide sequence of the *gyrB* gene. The nucleotide sequence of the wild-type (coumermycin A_1-sensitive) *B. bacilliformis gyrB* gene was determined from both DNA strands and is presented in Figure 1. Computer-assisted analysis of the *gyrB* gene showed a 2079-bp ORF. This ORF is characterized by a common initiation codon, ATG, that is preceded by putative -35 (TTCAAA) and -10 (GATAAT) consensus regulatory elements and a potential ribosomal binding site (AGTA) (Fig. 1).

Further analysis of the ORF indicated that the encoded protein had a deduced length

Figure 1. Nucleotide and predicted amino acid sequence of *B.bacilliformis gyrB*. The nucleotide sequence of a 2250-bp fragment containing the wild-type coumermycin A_1-sensitive *B. bacilliformis gyrB* is shown. Nucleotides within the 2079-bp ORF are given in uppercase letters and the deduced 692 residue amino acid sequence is shown below each corresponding codon. Putative consensus regulatory elements are indicated (-35, -10, Ribosomal Binding Site). The stop codon is marked with an asterisk. The three codons (and their corresponding amino acids) in which single nucleotide substitutions were found resulting in coumermycin A_1 resistance are boxed. The unusually long 52 residue N-terminus is typed in boldface. The predicted molecular mass of the mature protein is 77.5 kDa. The GenBank accession number for the *gyrB* gene is U82225.

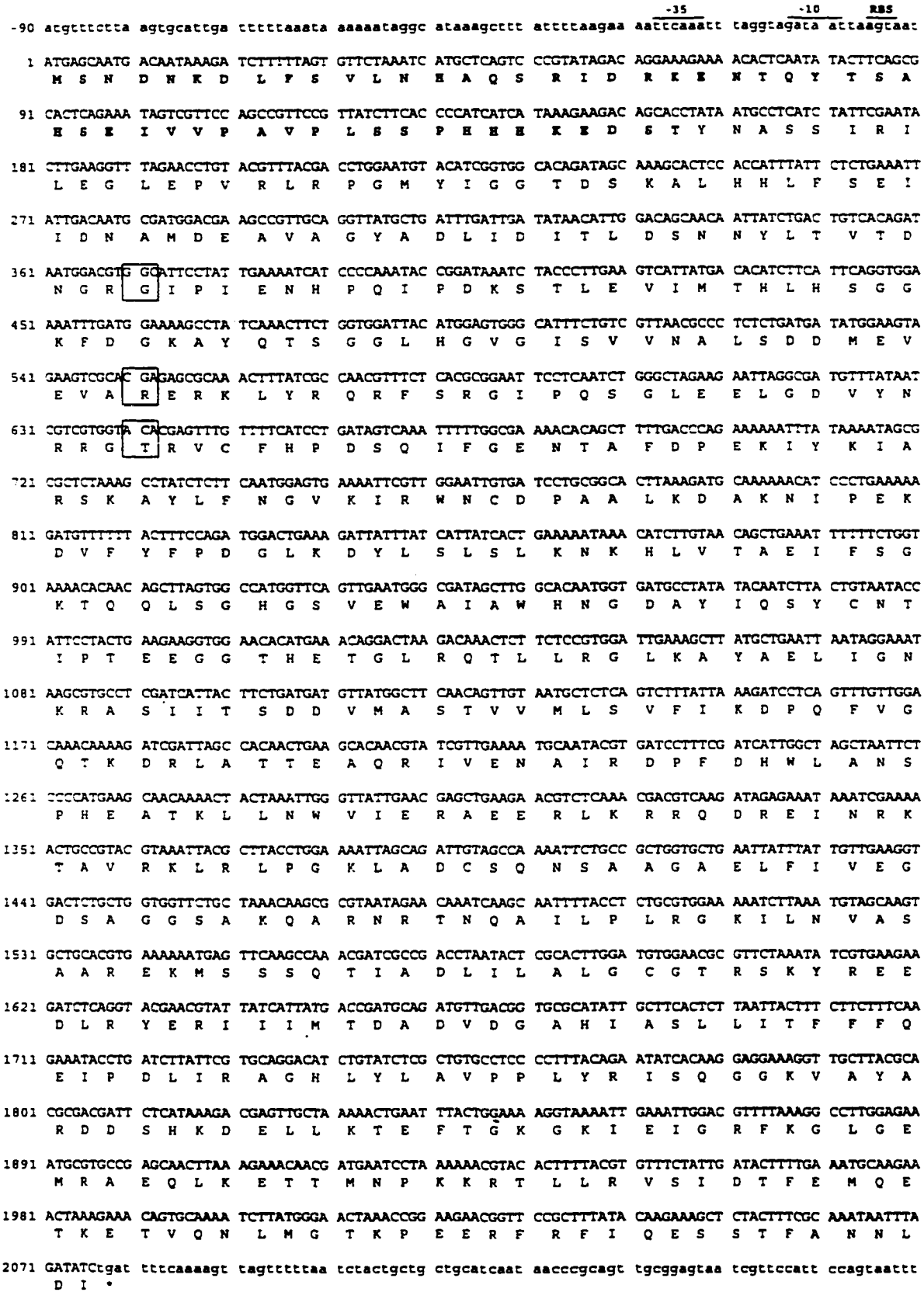
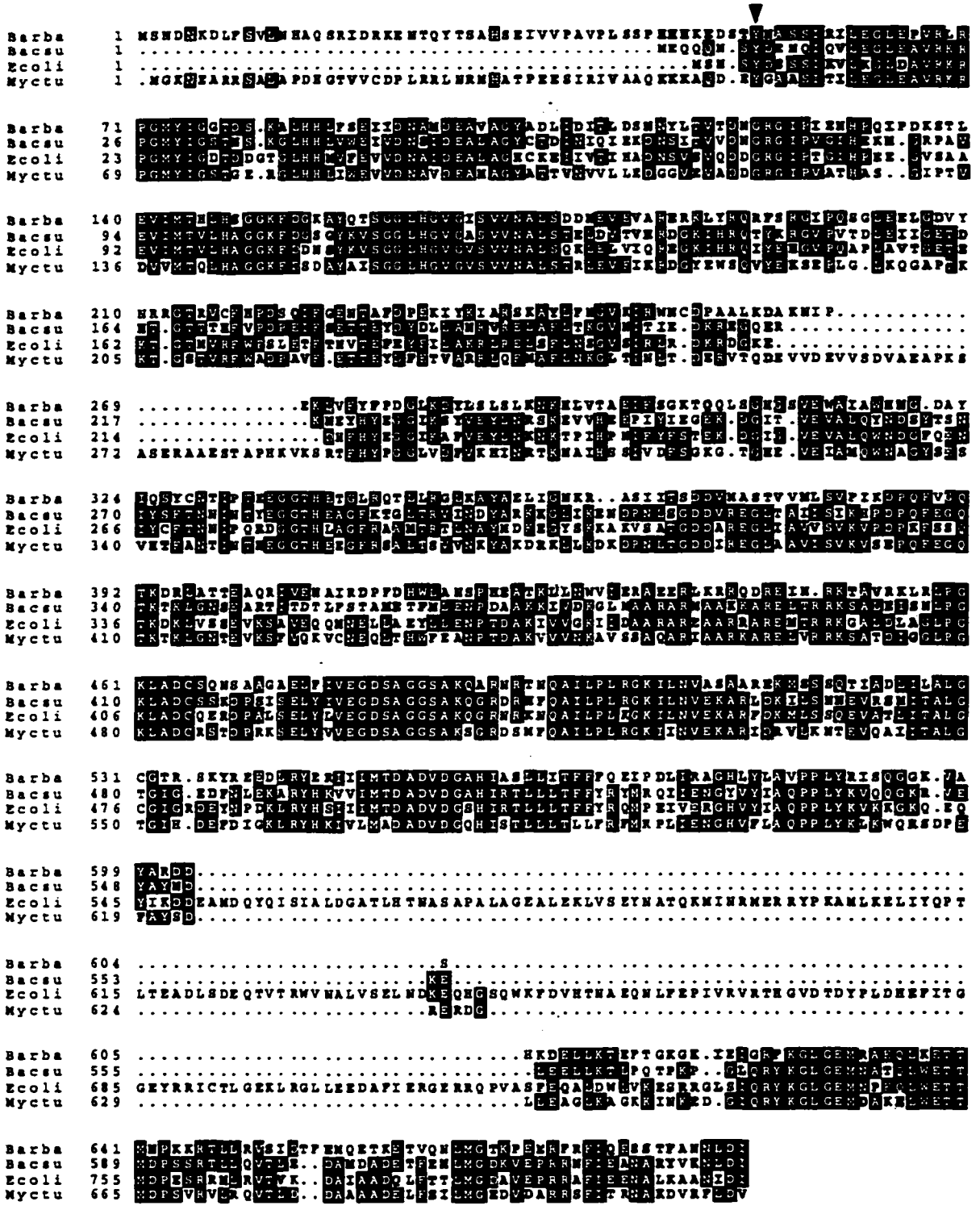


Figure 2. Multiple Alignment of *B. bacilliformis* GyrB with *B. subtilis E. coli* and *M. tuberculosis* GyrB. Multiple alignment of *B. bacilliformis* GyrB (Barba) with *B. subtilis* GyrB (Bacsu), *E. coli* GyrB (Ecoli), and *M. tuberculosis* (Myctb) generated with CLUSTAL-W 1.6 (171) and formatted with BOXSHADE 3.21 (76). Identical amino acid residues are shaded black, conserved residues noted in grey, and introduced gaps shown by dots. Note the unusual 53 residue N-terminal extension that is similar in length to the N-terminus of *M. tuberculosis* GyrB. The first universally conserved residue (*E. coli* of Tyr5) is indicated by the arrowhead. GenBank accession numbers for these sequences are U82225 (Barba GyrB), D26185 (Bacsu GyrB), AE000447 (Ecoli GyrB) and X78888 (Myctu GyrB).

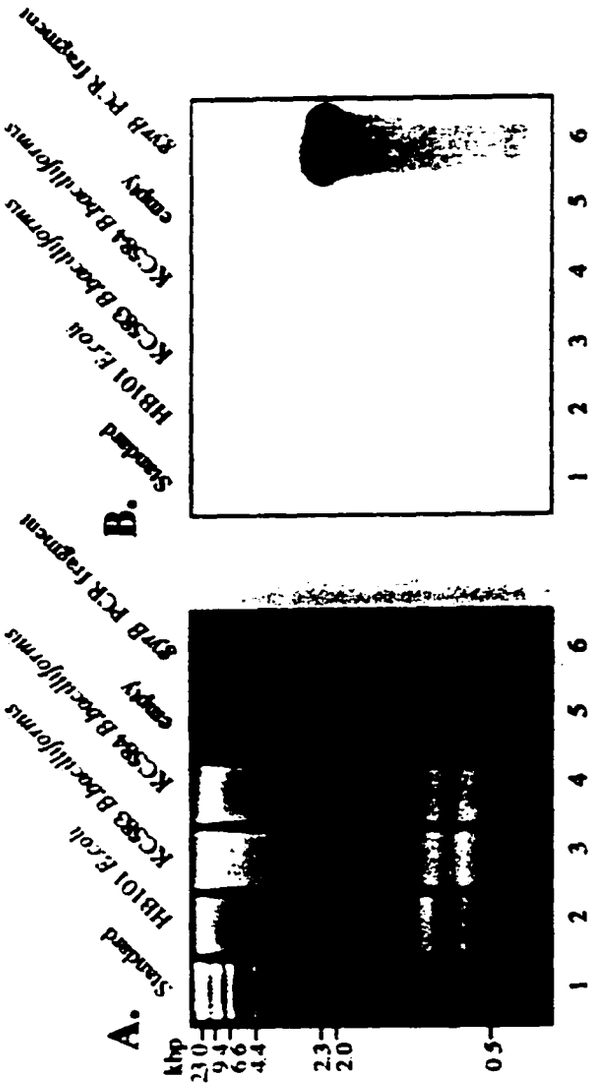


of 692 amino acid residues and a predicted molecular mass of approximately 77.5-kDa. BLAST (2) homology searches indicate that *B. bacilliformis* GyrB is most similar to *Bacillus subtilis* GyrB with an amino acid sequence identity of 40.1%. The *B. bacilliformis* subunit shares 34.1% identity with *E. coli* GyrB. Alignment of the deduced amino acid sequence from *B. bacilliformis gyrB* with the known amino acid sequences of GyrB's from *E. coli*, *B. subtilis*, and *Mycobacterium tuberculosis* (using Clustal-W 1.6) indicates multiple areas of strong homology (Fig. 2) and reveals that the *B. bacilliformis* GyrB has an unusually long N-terminus. Sequence analysis of ~600-bp of flanking sequence indicate a possible gene upstream of *gyrB* with homology to lipoate-protein ligase B, whereas 3' flanking sequence produces no areas of strong homology to database sequences (data not shown).

DNA hybridization analysis. In order to verify that the *gyrB*-containing fragment was of *Bartonella* origin, DNA hybridization analysis was done using *Bam*HI-digested DNA from *B. bacilliformis* strains KC583 and KC584 and from *E. coli* HB101. As shown in Figure 3B, Southern blots probed at high stringency (7% mismatch) with a ^{32}P-labeled 2410-bp PCR fragment derived from *B. bacilliformis* KC583 template (using amplimers GYRB-F and GYRB-R) clearly demonstrated single hybridization bands from both strains of *B. bacilliformis* (Fig. 3B, lanes 3 and 4). No signal was observed in *Bam*HI-digested *E.coli* HB101 DNA (Fig. 3, lane 2). In addition, the G+C content of the ORF (38.4 %) is in good agreement with the overall 39 mol % G+C of *B. bacilliformis* (8).

***In vitro* expression of *gyrB*.** To determine if *E. coli* transcriptional/translational machinery would express the cloned *B. bacilliformis gyrB*, an *E.coli* -S30 cell-free DNA expression kit (Promega) was used to produce polypeptides *in vitro*. SDS-PAGE analysis

Figure 3. Detection of the *gyrB* gene in the *B. bacilliformis* chromosome by DNA hybridization. (A) Ethidium bromide-stained agarose gel (1%, w/v) containing: 1, λ *Hin*dIII size standards; 2, *Bam*HI-digested chromosomal DNA of *E. coli* strain HB101; 3, *Bam*HI-digested chromosomal DNA of *B. bacilliformis* strain KC583; 4, *Bam*HI-digested chromosomal DNA of *B. bacilliformis* strain KC584; 5, no DNA; 6, 2410-bp PCR fragment containing the entire *B. bacilliformis gyrB* ORF. (B) The corresponding autoradiograph following DNA hybridization with the described 2410-bp PCR fragment labeled with ^{32}P-dCTP. The lanes are the same as in (A) Note the hybridization signal in both *B. bacilliformis* strains (lanes 3 and 4).



of proteins expressed from pGYRB3 revealed a protein product consistent with the predicted molecular mass for GyrB of 77.5-kDa that was not expressed from the pCR2.1-TOPO control by this system (data not shown). The 77.5-kDa protein was the largest protein encoded although additional insert-specific protein bands of approximately 68, 65, 52 and 38-kDa were observed and may have been produced by the *E.coli*-S30 extract from anomalous ORF's on the non-coding strand of pGYRB3 or may be degradation products.

Functional complementation analysis. Since the S30 extract expressed the cloned *gyrB*, An isogenic pair of *E. coli* strains first described by Menzel and Gellert (37) was used to evaluate the in vivo function of the cloned *B. bacilliformis gyrB*. *E. coli* strain N99 carries a wild-type *gyrB*, and strain N4177 has two *gyrB* mutations, which together confer a coumermycin A_1 -resistant (cou^R) and temperature sensitive (ts) phenotype. Growth of strain N4177 is permissive at 30°C but is restricted at 42°C unless a functional *gyrB* is supplied in *trans* to complement the cou^R-ts mutation. Therefore, we wanted to determine whether *B. bacilliformis gyrB* could functionally complement strain N4177. To address this question the *B. bacilliformis gyrB* recombinant pGYRB3 was introduced into strains N99 and N4177, selected at 30°C and subsequently replica-plated and separately incubated at both permissive (30°C) and restrictive (42°C) temperatures. The *B. bacilliformis gyrB* recombinant, pGYRB3, was shown to increase the growth rate of strain N4177 at 42°C by approximately 3-fold relative to negative controls (Table 3). The presence of plasmids or varied incubation temperature did not affect the relative growth rates of host strain N99. The pattern of growth for this analysis was consistent and reproducible, and shows that *B. bacilliformis gyrB* can functionally complement the cou^R-ts mutation of *E. coli* strain N4177.

Isolation of coumermycin A_1-resistant mutants. Background coumermycin A1-resistant mutants were observed 7 days after inoculation and occurred at a frequency of ~6x10-9 when selected in the presence of 0.1 μg/ml coumermycin A_1. After initial selection, mutant strains were cultured on heart infusion agar supplemented with 0.04 μg/ml coumermycin A_1. A total of twelve *B. bacilliformis* KC583 coumermycin-resistant (CR) mutants were selected in this manner and designated CR1 through CR12 (Table 1). In the absence of coumermycin-A_1, the growth rate and gross morphology of the CR colonies were indistinguishable from wild-type strains.

Coumermycin A_1 resistance is correlated with mutations in the *gyrB* gene. Genomic DNA was isolated from wild-type *B. bacilliformis* KC583 and the 12 coumermycin A_1- resistant mutants. The region of the *gyrB* gene encoding the N-terminal domain was amplified by PCR with LESION-F and LESION-R primers and subsequently sequenced with the LESION-F primer. Further analysis of these sequences revealed single nucleotide transitions at three separate loci that resulted in four distinct amino acid substitutions. First, in five of the 12 coumermycin A_1-resistant strains (CR1, CR2, CR6, CR8, and CR9), identical G-to-A transitions at base 370 of the 2079-bp ORF resulted in a deduced Gly124 → Ser substitution for all 5 strains. Second, four of the 12 resistant strains (CR4, CR7, CR11, and CR12) carried a G-to-A transition at base 550 that resulted in a deduced Arg184 → Gln substitution. The third loci at which lesions were detected occurred in the Thr 214 codon, in which two different transitions were observed with two distinct deduced substitutions; ACA-to-GCA resulted in a Thr214 →Ala substitution

TABLE 2. Genotypic and phenotypic analysis of *B. bacilliformis gyrB* mutants

Residue	Substitution	Frequency[a]	MIC[b, c]	Homologous GyrB lesions
Glycine 124	Serine	41.6	0.2	*B. burgdorferi* Gly 74 → Ser [47] *S. aureus* Gly85 → Ser [51]
Arginine 184	Glutamine	33.3	0.2	*E. coli* Arg136 → Leu, Cys, His, Ser [12], [14] *B. burgdorferi* Arg133 → Gly, Ile [46] *S. aureus* Arg144 → Ile [51] *Haloferax* Arg137 → His [23]
Threonine 214	Alanine	8.3	0.2	*B. burgdorferi* Thr162 → Ile [47] *S. aureus* Thr173 → Asn [51]
Threonine 214	Isoleucine	16.6	0.3	

[a] The frequency is expressed as a percentage of the 12 total isolates.

[b] The MIC is defined as the concentration in μg/ml which completely inhibited growth.
The MIC for wild-type KC 583 was 0.06 μg/ml.

[c] The MIC was determined for CR3, CR4, CR5, and CR9, which are representative of each of the mutant types.

TABLE 3. Complementation with *B. bacilliformis gyrB*

E. coli Strain	Growth at 42°C[a] with the following plasmids:		
	none	pCR2.1-TOPO	pGYRB3
N99	+++[b]	+++	+++
N4177	+/-[c]	+/-	+++

[a] Restrictive temperature for N4177 [37]
[b] Robust growth
[c] Slight growth

(CR3), whereas ACA-to-ATA resulted in a Thr214 →Ile substitution (CR5, CR10). These data demonstrate that background coumermycin A_1 - resistant mutants are correlated with specific and localized lesions in the *gyrB* gene. Table 2 summarizes several genotypic and phenotypic attributes of the coumermycin A_1 resistant (CR) strains.

***In vitro* coumermycin A_1 susceptibilities.** We assessed the antibiotic susceptibility of wild-type B. *bacilliformis* KC583 to coumermycin A_1 by using agar dilution techniques. At concentrations above 0.03 μg/ml, growth rates were noticeably decreased and above 0.06 μg/ml growth appeared to be completely inhibited. Thus, the MIC for KC583 was determined to be 0.06 μg/ml. One representative of each of the four different *gyrB* mutant types was assayed for coumermycin A_1 susceptibility. MIC's for mutant strains CR3, CR4, and CR9 were 0.2 μg/ml, whereas CR5 demonstrated a slightly higher level of resistance with a MIC of 0.3 μg/ml (Table 2).

DISCUSSION

We have described the first isolation and molecular characterization of background mutant strains conferring natural resistance to an antibiotic for any *Bartonella* species. Generation of the mutant strains was accomplished by exposure to inhibitory (0.1 μg/ml) levels of the DNA gyrase inhibitor coumermycin A_1, and occurred at a frequency of ~6×10^{-9}. Based upon amino acid sequence alignments *B. bacilliformis* GyrB belongs to the shorter 650-amino-acid size class represented by homologues of enzymes from *B. subtilis, Mycoplasma pneumoniae, Staphylococcus aureus, Borrelia burgdorferi*, and

Haloferax (24). In the larger 800-amino-acid size class, represented by *E. coli* , an extra 150-amino acid block is found in the C-terminal domain of the protein (24) (Fig. 2). The commonly recognized ATP binding motif of GXXGXG is found at positions 162 to 167 of *B. bacilliformis* GyrB, corresponding to positions 114 to 119 of *E. coli* GyrB.

The structure of the *B. bacilliformis* GyrB is unusual in two ways. First, when analyzing GyrB's sequenced to date, the first N-terminal amino acid that demonstrates universal conservation throughout bacteria is a Tyr residue represented by *E. coli* Tyr5, corresponding to Tyr53 of *B. bacilliformis* (Fig. 2). The side chain of Tyr5 hydrogen bonds to the bound ATP analog (57). The number of amino acids preceding this conserved Tyr is less than 13 residues for nearly all bacteria examined to date. *B. bacilliformis* GyrB is unusual in this respect in that 52 amino acid residues precede the *E. coli* Tyr5 homolog, making it the longest N-terminal extension reported to date. Only *M. tuberculosis* has an N-terminal extension of this magnitude, with 50 amino acids (Fig. 2); however, the two extensions are not homologous. The crystal structure of *E. coli* GyrB N-terminal domain complexed with a non-hydrolyzable ATP analog shows that the N-terminal 13 residues form a protrusion that interacts with the other GyrB protomer (57). This interaction stabilizes the dimer interface and forms part of the ATP binding site (57). However, the N-terminus is apparently not ordered in the co-crystal structure with the coumarin inhibitor novobiocin (33). The function of the unusually long N-terminal extensions of *M. tuberculosis* and *B. bacilliformis* GyrB's is intriguing and remains to be determined. A second primary structural feature of *B. bacilliformis* GyrB that we have noted is Glu128 (*E. coli* Gly81 equivalent). In all wild-type GyrB's reported thus far, this residue is either glycine or aspartate, with the exception of the *Mycobacteria*, which have alanine or glutamate at this position. In this respect, *B. bacilliformis* GyrB is also more similar to the *Mycobacteria* GyrB. This position is one of three loci that is mutated in a

novobiocin-resistant *Haloferax* (Asp82 → Gly) (23), although it is distant from the coumarin binding site (33). Although both *B. bacilliformis* and *M. tuberculosis* are slow-growing bacteria and have several similar GyrB structural features, the effect of these properties on interactions with ATP or coumarins is unknown.

The mechanism of coumermycin A_1-resistance in *B. bacilliformis* mutants was identified by sequencing PCR fragments generated with primers amplifying the portion of the *gyrB* gene that encodes the N-terminal domain. We have isolated 12 coumermycin A_1-resistant mutants and have identified single nucleotide transitions at three separate loci resulting in single amino acid substitutions in the N-terminal domain of the GyrB protein. Lesions detected in the resistant *B. bacilliformis gyrB* genes are analogous in location and residue substitution to previously characterized resistant *gyrB* genes (12, 14, 23, 46, 47, 51). The crystal structure has revealed important interactions for each of the lesion sites. First, the side group of the *E. coli* Arg136 residue (*B. bacilliformis* Arg184) makes critical hydrogen bonds with the coumarins and with *E. coli* Tyr5 (*B. bacilliformis* Tyr53) on the other protomer (which is involved in ATP binding) (33). The second and third residues associated with coumarin resistance, *E. coli* Gly77 (*B. bacilliformis* Gly124) and *E. coli* Thr165 (*B. bacilliformis* Thr214), specifically interact with each other as well as stabilize interactions with ATP and coumarins (33).

These data demonstrate that the *B. bacilliformis* DNA gyrase B protein is a target for coumarin antibiotics. At a MIC of 0.06 μg/ml, wild-type *B. bacilliformis* was shown to be more susceptible to growth inhibition by coumermycin A_1 than almost all other bacteria tested (59), and is 250 times more susceptible than *E. coli* (19). These data are consistent with the finding that *Bartonella* are extremely susceptible to a variety of antibacterial agents *in vitro* (34). The mutant strains demonstrated a ~5 fold increase in resistance levels. GyrB mutants represented by strains CR3, CR4, and CR9 were

determined to have a MIC of 0.2 μg/ml, whereas the MIC of CR5 was 0.3 μg/ml. This suggests that a Thr214 → Ile substitution confers a higher level of resistance than Thr214 → Ala, Gly124 → Ser or Arg184 → Gln, consistent with findings in *B. burgdorferi* (47).

The transition between the diverse thermal environments of the arthropod vector and the human host, as well as the presentation of the verruga peruana on the extremities (<37°C) suggests that there is a close relationship between temperature and gene expression in *B. bacilliformis*. *Yersinia enterocolitica* DNA gyrase mutants simulate thermo-induced alterations of DNA supercoiling with coincident phenotypic changes (45). Likewise, DNA topology regulated by DNA gyrase may play an important role in the survival or virulence of *B. bacilliformis* in both the vector and host. DNA gyrase mutants may provide a method for analysis of thermoregulation. Furthermore, this report may contribute toward the design of novel antibacterial and antitumor agents that target DNA topoisomerases.

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CHAPTER FIVE

General Discussion

A system for genetic manipulation greatly facilitates investigation of the poorly understood virulence characteristics of the *Bartonella*. The overall goal of this project was to develop a system of genetic manipulation for *Bartonella bacilliformis* and to use this system to satisfy molecular Koch's postulates for the flagellum, a putative virulence factor. First, we hypothesized that a system for genetic manipulation could be developed and approached this hypothesis by experimenting with two separate loci of *B. bacilliformis*: the *gyrB* gene and the *fla* gene.

After several initial attempts at mutagenizing wild type *B.bacilliformis* with suicide plasmids, it became apparent that there were obstacles hindering *in vivo* homologous recombination. This initiated an investigation into methods that alleviated restriction as well as biochemical and metabolic manipulations that have been shown to increase the likelihood of homologous recombination. Chapter Two summarized these strategies and discussed the two protocol alterations that were used in combination to inactivate the *fla* gene by electroporation-mediated introduction of the suicide vector pUB508.

The first, and most important, protocol alteration concerned the host strain of *B. bacilliformis* used for genetic manipulation studies. Several attempts at repeating the plasmid transformation described by Grasseschi and Minnick (61) with the cosmid pEST produced no transformants in KC584 strain of *B. bacilliformis*. It was learned, however that during these experiments (personal communication, M.F. Minnick, 1996), the cultures were continually passaged, divergent from the low passage KC584 strain of *B. bacilliformis* that we were using as a host strain. Although this protocol variation seemed minor, with the encouragement of Dr. George Card (personal communication, Dr. George Card, 1996), we investigated this possibility.

We started with a stock of the pEST-transformed strain of *B. bacilliformis* extant in the laboratory, designated HG584 (Helen Grasseschi) for this study. pEST is a replicative plasmid that harbors the resistance cassette *neomycin phosphotransferase I* and confers kanamycin resistance to HG584. Clonal subcultures of strain HG584 were passaged three times without antibiotics. Kanamycin-sensitive clones were then verified for pEST curing by Southern analysis and PCR. The resulting strain, PCB or 'pEST cured *Bartonella* ' was renamed JB584. Strain JB584 demonstrated a 2000-fold increase in 'plasmid' transformation efficiencies with the plasmid pBBR1MCS-2 compared to KC584 and was successfully used as the host strain for genetic site-directed manipulation studies.

To illustrate the apparently serendipitous nature of the isolation of strain JB584 (or rather the natural mutation(s) that arose after repeated subculture by H. Grasseschi), prior to this finding, another high passage strain of *B. bacilliformis* was obtained from L. Hendrix (Texas A&M University). This strain, termed *KC584-long passage*, was determined to be methylase minus based on the ability of normally non-recognizing, non-digesting restriction enzymes to fragment chromosomal preparations. One would expect a methyl-minus mutant to accordingly be restriction-minus, for self would be recognized as non-self, and genomic fragmentation would result in death. Unfortunately, the *KC584-long passage* strain inhibited plasmid transformation attempts similar to the low-passage wild-type KC584. In contrast, strain JB584 is apparently methylase-positive.

The natural mutation(s) of strain JB584 that permits high transformation efficiencies is still unknown. Based on numerous unsuccessful attempts of genetic manipulation using linear gene targeting constructs with both the *fla* and *gyrB* loci, we believe there is still a powerful exonuclease activity in JB584. High passaged strains of *B. henselae*, for example, have shown a reduction in *in vitro* pathogenicity (13). Thus, the integrity of JB584 must be questioned in this light, for it is also a high passage strain. Considering the adhesion data generated in Chapter Three of this work, JB584 does not seem to have a

reduction in adhesion when compared to the low-passaged wild-type strain KC584. However, virulent clinical isolates of *B. bacilliformis* have not been compared to KC584 in *in vitro* pathogenicity assays. Much can be learned from the utilization of the transformation-competent strain JB584, but effects of passage cannot be realized until further virulence analyses are done. One interesting use for JB584 is as a DNA modifying strain. Considering the methylase positive phenotype, coupled with strain transformation competency, plasmids could conceivably be introduced into JB584, methylated, purified, and subsequently introduced into a virulent clinical isolate strain. In conclusion, we hypothesize that the high transformation efficiencies associated with strain JB584 are a result of a restriction endonuclease mutation. Several attempts to clone and characterize the methylase-restriction enzymes of *B. bacilliformis* using a blue-white isolation system proved unsuccessful.

The second protocol alteration that may have influenced the efficiencies of homologous recombination was the addition of low levels of methionine to the recovery broth. Since strain JB584 apparently did not fragment foreign supercoiled plasmids, we hypothesized that we could encourage the *in vivo* conditions required for homologous recombination to occur. This hypothesis was based on increasing the *in vivo* levels of RecA. Although RecA-independent homologous recombination has been demonstrated (16), RecA is considered the mediator of most prokaryotic homologous recombination. Growth in the presence of methionine has been shown to decrease the levels of S-adenosylmethionine (SAM) *in vivo* (78, 165). SAM is the methyl donor for DNA-methylases and is also required for the function of Type I restriction enzymes (17). We hypothesized that in the presence of methionine a reduction in SAM levels would inhibit normal methylation of DNA *in vivo*. Directly, this should create a confusion between self and non-self DNA allowing foreign DNA, such as a suicide plasmid, to survive long enough to find its target and cross over. Indirectly, a confusion over self and

non-self would hypothetically induce self-restriction and thus, the generation of single-stranded DNA. Single-stranded DNA is the signal for SOS induction, and induction of SOS results in an increase in RecA. Thus, we hypothesize that methionine is inhibiting DNA methylation, which distorts self vs. non-self recognition and results in single stranded DNA, inducing SOS and finally resulting in an increase in RecA. Furthermore, since UV light-induced SOS induction has been correlated with temporary alleviation of host-controlled restriction in *Cyanobacterium*, *Bacillus*, and *E. coli* (47, 74, 75, 178, 187), it follows that a non-mutagenic method of SOS induction should decrease restriction. The presence of methionine does not seem to have an effect on rates of plasmid transformation, but in light of the benign enhancements to homologous recombination, we have left this supplement in our protocol. The efficacy of methionine as a nonspecific, non-mutational (ie. UV-light) SOS inducer to enhance homologous recombination is unknown.

With the two protocol modifications of JB584 and methionine we were able to construct a *fla* -minus mutant of *B. bacilliformis*. This was accomplished by site-directed mutagenesis using the suicide vector, pUB508. The *fla*- mutant [JB585] was unable to synthesize flagellin, and a motility assay demonstrated that the mutant was non-motile. We then complemented this *fla* -minus mutant by introducing the wild type *fla* ORF on the shuttle plasmid pBBRFLAG. The complemented mutant was able to express, assemble, and secrete flagellin subunits resulting in a motility phenotype that was indistinguishable from wild-type. Finally, the strains were assessed for their ability to adhere to human erythrocytes. The *fla* mutant demonstrated a significant loss in its ability to adhere, whereas the complemented mutant showed a partial restoration of its ability to adhere to human erythrocytes.

Pathogenicity is defined as the ability of an organism to cause disease. The tools or mechanisms that the organism uses to cause disease are defined as the virulence factors.

These virulence factors, or virulence determinants, are best studied by the satisfaction of a molecular set of Koch's postulates (49). In 1875 Robert Koch established a set of experiments, now termed Koch's postulates, which enabled him to prove that a germ, *Bacillus anthracis*, was the causative agent of the disease anthrax. With these experiments, Koch verified the germ theory of disease. In 1926 Noguchi and Battistini demonstrated Koch's postulates with *B. bacilliformis* in macaque monkeys (180). To date, many infectious agents have been discovered as the cause of disease by verifying Koch's postulates. Recently, molecular Koch's postulates was proposed by Falkow (49) as a precise means of determining the relationship between a putative virulence factor and the pathogenesis of an organism. These postulates are mentioned in Chapter Three. By demonstrating the significant reduction in the ability of the *fla* -minus mutant to adhere to human erythrocytes, combined with the partial restoration of adherence phenotype expressed by the complemented strain, we have at least partially demonstrated molecular Koch's postulates for the *B. bacilliformis* flagellum. This is the first demonstration of molecular Koch's postulates for any *Bartonella* as well as the first demonstration of site-specific mutagenesis and complementation for the *Bartonella*.

The mechanism by which *Bartonella* gain entry into erythrocytes is unknown. The working model of *B. bacilliformis* erythrocyte invasion is termed 'forced endocytosis', in which the organism uses several virulence mechanisms to adhere and subsequently gain entry into the erythrocyte (119). Initial interaction and adhesion with the erythrocyte surface may be influenced by the flagella (164), filament A (121), or BFP (114). Subsequently, the activity of three virulence factors, IalA/IalB (132), deformin (116, 189), and the flagella (14, 164) may work together to coordinate erythrocyte invasion. Our study concerning the flagella-mediated erythrocyte adhesion supports this model of 'forced endocytosis'.

The system of genetic manipulation developed herein will provide the genetic tools

to decipher much more about the pathogenesis of *Bartonella*. One example of this is the recent demonstration of site-specific mutagenesis of the *ialB* locus (personal communication, S. Coleman, 1998). Another example is the research currently being conducted in our laboratory concerning genetic exchange by the bacteriophage-like-particle (BLP) associated with *B. bacilliformis* (personal communication K. Barbian and M.F. Minnick). As mentioned in chapter one, this phage packages random 14-kbp fragments of chromosomal DNA. By purifying the BLP's associated with insertional mutants of *B. bacilliformis*, one can study transduction based on the ability of these particles to deliver resistance cassettes. Finally, a strain of *B. bacilliformis* has been constructed that is expressing the green fluorescent protein (GFP)(176) from the *B. bacilliformis fla* promoter. This strain can be used for numerous applications including *in vivo* localization in both host and vector, as well as for studies on regulation of flagellin expression.

We believe that this system of genetic manipulation will contribute significantly to the understanding of the molecular biology and pathogenesis of *Bartonella*.

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IMAGE EVALUATION
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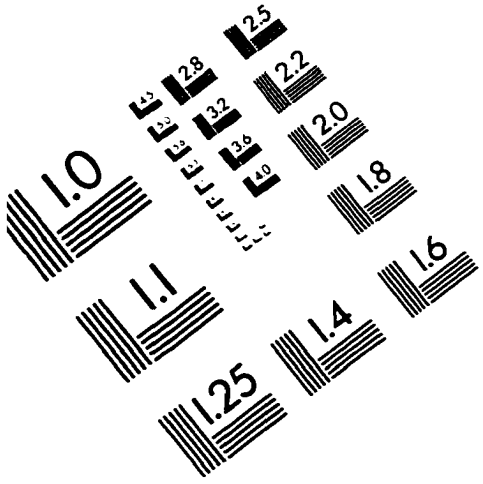

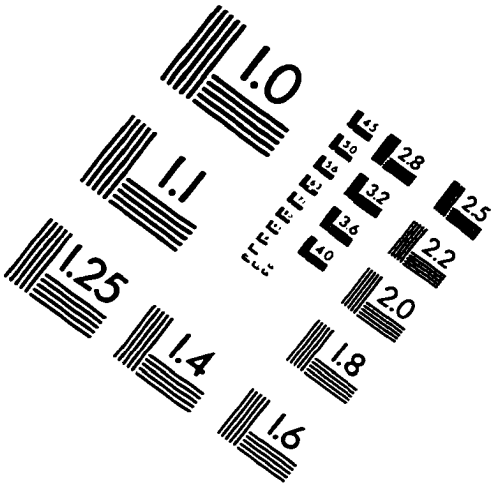

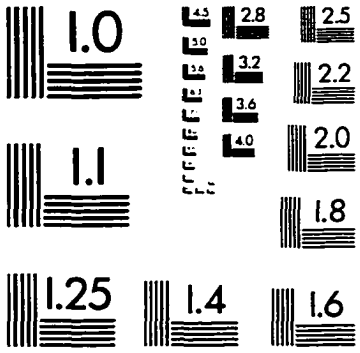

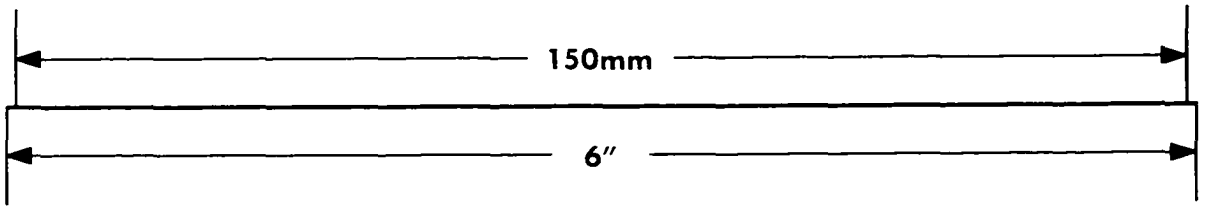

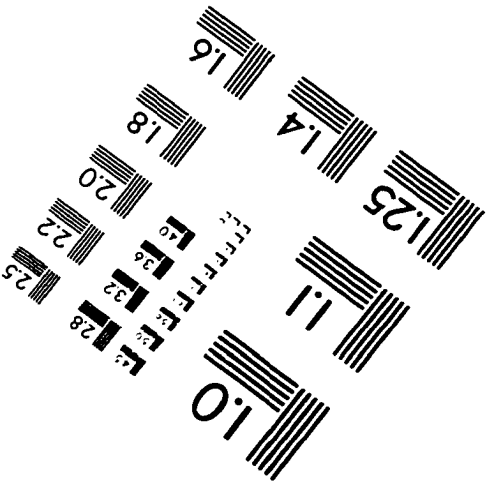

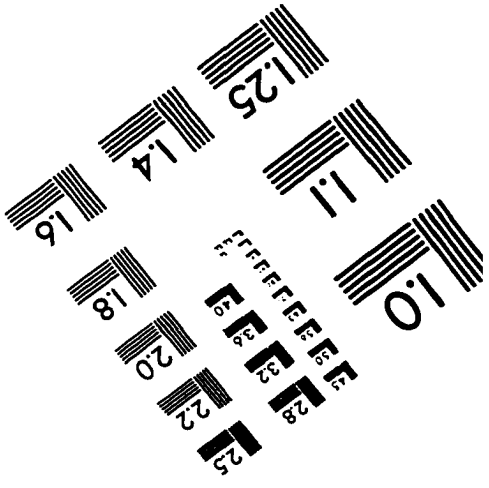

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